

Neuroscience

Research and textbook

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Author in Chief: Aliasghar Tabatabaei Mohammadi

Gmail: Dr.Alitabatabaei98@gmail.com

Urmia University of Medical Sciences

Authors

1. Aliasghar Tabatabaei Mohammadi

Gmail: Dr.Alitabatabaei98@gmail.com

Urmia University of Medical Sciences

2. Shakiba Tolou-Shikhzadeh-Yazdi

Affiliation: Department of Biology, Faculty of Sciences, Islamic Azad University, Mashhad,
Iran

Gmail: Shakibatolou1997@gmail.com

3. Farideh Salimi Bajestani

Affiliation: SABZEVAR UNIVERSITY OF MEDICAL SCIENCES

Gmail: frdslm360@gmail.com

4. Zeinab Haghani

Affiliation: Department of psychology Islamic Azad University, science and research
Tehran, Iran

Gmail: Zeyhaghani@gmail.com

5. Mahsa salehi

Affiliation: Department of Basic Science Faculty of Veterinary Medicine
Shahid Bahonar University of Kerman

Gmail: Mahsa.s4572@gmail.com

6. Samin Saeedpour Aval

Affiliation: Department of Faculty of Technical Engineering,
Islamic Azad University, Sari, Iran

Gmail: saminsaeedpour@gmail.com

7. Fateme Darijani

Affiliation: Department of psychology and educational sciences
Islamic Azad University of Zarand,Kerman,Iran

Gmail: Fatemedarijani7471@gmail.com

8. Seyedeh Ghazal Asiaee

Affiliation: Department of medicine,
Qazvin University of Medical Science, Qazvin, Iran
Gmail: Ghazalasiaee1370@gmail.com

9. Fatemeh Zivari

Affiliation: Department of General and Clinical Psychology, Allameh Tabataba'i University,
Public university, Tehran, Iran
Gmail: fa.zivari@gmail.com

10. Hadise Rezayi

Affiliation: Department of psychology, Payame noor mashhad Mashhad, Iran
Gmail: drhadisrezayi992@gmail.com

11. Ilia Asadi

Affiliation: Student Research Committee, Semnan University of Medical Sciences, Semnan, Iran
Gmail: ilia.asadi@yahoo.com

12. Khashayar Bahramsari

Affiliation: Student Research Committee, Semnan University of Medical Sciences, Semnan, Iran
Gmail: Kbahramsari@yahoo.com

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Chapter1

Mitochondria are highly energetic organelles that play fundamental regulatory roles in multiple cellular events, from bioenergetics to oxidative stress, Ca^{+2} signaling/homeostasis and metabolism. To manage these complex functions, mitochondria undergo many dynamic changes; they can associate with cytoskeleton and move to deliver energy, in the form of ATP, or metabolites where they are needed; they can also undergo cycles of fission and fusion to manage metabolic challenges associated with specific environmental conditions (McBride et al., 2006).

Maintaining mitochondrial functioning is crucial for cellular health, and mitophagy, the selective removal of damaged mitochondria, is emerging as a fundamental cellular strategy to eliminate dysfunctional mitochondria and allow the cell to replenish the pool of healthy mitochondria *via* mitochondrial biogenesis (Roca-Portoles and Tait, 2021). This process is of particular importance in neurons

because neurons have high metabolic demands and, as post-mitotic cells, cannot “dilute” damaged mitochondria through cell division ([Evans and Holzbaur, 2020b](#)). Importantly, mitochondrial dysfunction and mitophagy defects have been linked to many neurodegenerative disorders ([Cai and Jeong, 2020](#); [Cen et al., 2021](#); [Li et al., 2021](#); [Mani et al., 2021](#); [Wang X.-L. et al., 2021](#)).

Functional overview of PTEN-induced kinase

1/Parkin dependent mitophagy

PTEN-induced kinase 1 (PINK1) is a mitochondria-localized serine/threonine kinase that is present at low levels under basal conditions but is stabilized and accumulates on the outer mitochondrial membrane (OMM) as a result of mitochondrial damage and/or depolarization ([Matsuda et al., 2010](#); [Narendra et al., 2010](#); [Rakovic et al., 2010](#); [Vives-Bauza et al., 2010](#)). At steady state (normal conditions), the mitochondrial targeting signal (MTS) directs PINK1 to the mitochondria where it transverse the OMM, *via* Translocase of the OMM 40 (TOM40), and the inner mitochondrial membrane (IMM), *via* Translocase of IMM 23 (TIM23) ([Wang N. et al., 2020](#)). The positive charge on the MTS allows it to translocate into the matrix where it is cleaved by Mitochondrial Processing Peptidase (MPP) ([Wang N. et al., 2020](#)). Subsequently, PINK1 is cleaved by

Presenilins-Associated Rhomboid-Like protein (PARL) in the IMM causing PINK1 release into the cytoplasm where it is degraded by the ubiquitin proteasome system ([Figure 1A](#); [Wang N. et al., 2020](#)). MPP and PARL play crucial roles in maintaining homeostatic PINK1 localization as inhibition of either MPP or PARL results in abnormally high levels of mitochondrial PINK1 that are sufficient to stimulate the induction of mitophagy ([Shi et al., 2011](#); [Greene et al., 2012](#); [Meissner et al., 2015](#)). When mitochondria are damaged and/or depolarized, failure to fully import PINK1 into the matrix disrupts homeostatic PINK1 processing with consequent accumulation and dimerization on the mitochondria surface ([Figure 1A](#); [Sekine et al., 2019](#); [Wang N. et al., 2020](#)). The dimerization causes PINK1 to undergo autophosphorylation at Ser228 and Ser402; phosphorylated PINK1 can then phosphorylate ubiquitin and OMM-associated proteins, including Miro, TRAP1 and MFN2, as well as engage Parkin ([Figure 1A](#); [Trempe and Fon, 2013](#); [Aerts et al., 2015](#); [Tanaka, 2020](#); [Wang L. et al., 2020](#)). The accumulation of PINK1 on the mitochondria surface stimulates the induction of mitophagy while proteolytic processing and release of PINK1 from the mitochondria surface has the opposite effect ([Shi et al., 2011](#); [Greene et al., 2012](#); [Meissner et al., 2015](#)).

The E3 ubiquitin ligase Parkin resides in the cytosol in a closed and inactive conformation under basal conditions; it is activated and recruited to the

mitochondria in response to depolarization-induced accumulation of PINK1 on the OMM and its subsequent kinase activity ([Geisler et al., 2010a](#); [Matsuda et al., 2010](#); [Rakovic et al., 2010](#); [Vives-Bauza et al., 2010](#); [Tang et al., 2017](#)).

Phosphorylated ubiquitin chains on OMM proteins resulting from PINK1 activity serve as receptors for Parkin recruitment to the mitochondria ([Shiba-Fukushima et al., 2014](#); [Okatsu et al., 2015](#)). Binding to phosphorylated ubiquitin induces Parkin conformational change to the “open” intermediate state where the interaction between the ubiquitin-like (UBL) and the RING1 domains is disrupted ([Ham et al., 2016](#); [Tanaka, 2020](#); [Wang N. et al., 2020](#)). Under this conformation, the UBL is separated from the core and is readily accessible for PINK1-mediated phosphorylation at Ser65 resulting in the fully activated form of Parkin ([Shiba-Fukushima et al., 2014](#); [Tang et al., 2017](#); [Tanaka, 2020](#); [Wang N. et al., 2020](#)). Activated Parkin has been shown to catalyze the formation of ubiquitin chains linked by Lys6, Lys11, Lys27, Lys48, and Lys63 ([Figure 1B](#); [Geisler et al., 2010a](#); [Bader and Winklhofer, 2020](#); [Tanaka, 2020](#)). This creates a positive feedback loop that amplifies PINK1/Parkin signaling as Parkin-driven ubiquitination of OMM proteins creates more sites for PINK1 phosphorylation, which can then recruit more Parkin. MFN1/2, VDAC1/2/3, Miro, HK1/2, TOMM20, TOMM70A, RHOT1/2, FAF2, and CISD1/2 have all been shown to be

ubiquitinated by Parkin (Springer and Macleod, 2016; McLelland et al., 2018; Bader and Winklhofer, 2020).

With ubiquitin binding domains and the ability to recruit the cytosolic components of the autophagy machinery, mitophagy receptors ensure recognition and degradation of damaged mitochondria. Autophagy protein 32 (ATG32) is the only known mitophagy receptor in yeast whereas mammals have several mitophagy receptors including NDP52, OPTN, TAX1BP1, NBR1, and -perhaps- p62 (Kanki et al., 2009; Wong and Holzbaur, 2014a; Evans and Holzbaur, 2020b; Montava-Garriga and Ganley, 2020). The ubiquitin-binding capability of NDP52, OPTN, TAX1BP1, and p62 receptors increases in response to TBK1 phosphorylation, and inhibition of TBK1 activity reduces autophagosome formation (Moore and Holzbaur, 2016; Bader and Winklhofer, 2020; Evans and Holzbaur, 2020b; Montava-Garriga and Ganley, 2020; Wang L. et al., 2020). Mitophagy receptors have confirmed LC3-interacting regions (LIR) suggesting that they can independently recruit LC3 β (Figure 1C). However, recent work has highlighted the role of ULK1 complex recruitment as mitophagy is still active in cellular systems where the LC3/ATG8 conjugating system is inactivated (Montava-Garriga and Ganley, 2020; Wang L. et al., 2020). NDP52 directly binds FIP200, a member of the ULK1 complex, but it is currently unknown whether OPTN or TAX1BP1 interact with the ULK1 complex (Montava-Garriga and Ganley, 2020; Wang L. et

al., 2020). The different mitophagy receptors have redundant functionality as OPTN, NDP52, and TAX1BP1 all localize to the mitochondria after depolarization or localized ROS generation on the same timescale (Moore and Holzbaur, 2016). However, despite the apparent functional overlap, unique consequences are associated with differential expression of the receptors. The expression of OPTN seems to be of particular importance. Indeed, overexpression of OPTN or NDP52 on a knock-out background of five mitophagy receptors produces the greatest rescue, while deletion of OPTN reduces the speed of mitochondrial engulfment by the autophagosome to a greater extent than NDP52 deletion (Moore and Holzbaur, 2016; Evans and Holzbaur, 2020b). Engagement of LC3 β and consequent activation of the cytosolic autophagy machinery, leads to the engulfment of mitochondria into mitophagosomes. Mitophagosomes then fuse with lysosomes and lysosomal proteases degrade the mitochondria (Figure 1D). Additional mechanistic information on the activation and regulation of mitophagy can be found elsewhere (Jishi and Qi, 2022; Li et al., 2022).

Mitophagy and neurodegenerative diseases

The identification of disease-causing mutations in multiple genes associated with the induction and progression of mitophagy underscores the biological importance of ensuring efficient clearance of dysfunctional (and perhaps even excessive)

mitochondria within the cell. Interestingly enough, the great majority of these mutations appear to affect the nervous system. For example, mutations in PINK1 and Parkin are associated with early-onset forms of Parkinson's Disease (PD) ([Borsche et al., 2021](#); [Vizziello et al., 2021](#)) while mutations in MFN2 are associated with axonal forms of Charcot-Marie-Tooth disease 2 (CMT2) and hereditary motor and sensory neuropathies (HMSN) ([Zaman and Shutt, 2022](#)). Finally, mutations in OPTN have been linked to primary forms of glaucoma ([Sears et al., 2019](#)) as well as amyotrophic lateral sclerosis (ALS) ([Benson et al., 2021](#)). In this section, we will describe evidence that supports causative association between different neurodegenerative diseases and dysfunctional mitophagy. We recognize that in some cases, such as with PD-associated mutations in PINK1 and Parkin, the association is direct, while in others, such as with Alzheimer's disease (AD), the association is only indirect. However, whether defective mitophagy is the cause or the consequence of the disease, it is still important to evaluate its immediate pathologic role and dissect possible disease-mitigating approaches.

Mitophagy dysfunction in Parkinson's disease

PD is a neurodegenerative disease clinically characterized by bradykinesia with rest tremor and/or rigidity ([Jankovic and Tan, 2020](#); [Bloem et al., 2021](#)).

Pathologically, the brains of PD patients display protein aggregates, referred to as

Lewy bodies and typically enriched in α -synuclein, and degeneration of nigrostriatal dopaminergic cells ([Blauwendraat et al., 2020](#); [Bloem et al., 2021](#)). The resulting imbalance between excitatory and inhibitory input into the basal ganglia causes bradykinesia whereas degeneration of non-dopaminergic pathways is associated with non-motor PD symptoms ([Jankovic and Tan, 2020](#); [Bloem et al., 2021](#)). The incidence of PD increases with age. More than 10 million individuals are currently affected by PD worldwide; however, this number is expected to rise significantly due to changes in population age distribution as well as improved diagnostic tools ([Blauwendraat et al., 2020](#); [Jankovic and Tan, 2020](#); [Bloem et al., 2021](#)).

Monogenic PD accounts for roughly 3–5% of all cases, and several genes have been identified with high confidence to cause PD ([Blauwendraat et al., 2020](#); [Bloem et al., 2021](#)). Though single gene mutations are rare, mitochondrial morphological and functional phenotypes found in patient tissue and in patient derived cell lines are generally conserved in patients with familial and sporadic PD. Decreased fusion and increased fission protein levels, as well as imaging studies quantifying mitochondrial length and branching, support increased mitochondrial fragmentation in PD ([Zilocchi et al., 2018](#); [Walter et al., 2019](#); [Yakhine-Diop et al., 2019](#); [Hanss et al., 2021](#)). These same studies also reveal more spherical, swollen mitochondria with less cristae suggesting

accumulation of damaged mitochondria ([Zillocchi et al., 2018](#); [Walter et al., 2019](#)). Functional studies support these findings as mitochondria in patient-derived cell lines have reduced mitochondrial membrane potential (MMP), ATP production, and respiration as well as increased ROS ([Grünewald et al., 2010](#); [Hsieh et al., 2016](#); [Delgado-Camprubi et al., 2017](#); [Walter et al., 2019](#); [Yakhine-Diop et al., 2019, 2021](#); [Wauters et al., 2020](#); [Hanss et al., 2021](#)). Mitochondrial morphological and functional phenotypes have been demonstrated in a wide range of PD-like animal and cell models, including increased fragmentation, disrupted cristae structure, depolarized membrane potential, reduced ATP production, decreased respiration, and increased generation of ROS ([Lesage et al., 2016](#); [Villeneuve et al., 2016](#); [Zhang et al., 2016](#); [Chen et al., 2018](#); [Martinez et al., 2018](#); [Chiu et al., 2019](#); [Li et al., 2019](#); [Anand et al., 2020](#); [Liu et al., 2020](#); [Noda et al., 2020](#); [Erustes et al., 2021](#)). A summary of mitochondria alterations associated with PD are listed in [Table 1](#).

Monogenic, autosomal recessive PD is strongly associated with defective mitophagy as the two most commonly mutated genes are *PARK2*, the gene encoding Parkin, and *PINK1* (in order of prevalence) ([Jankovic and Tan, 2020](#); [Wang N. et al., 2020](#)). PD-associated mutations in *PINK1* and *PARK2* have all been shown to inhibit mitophagy, although individual mutations appear to disrupt different steps of the process ([Geisler et al., 2010b,a](#); [Lee et al.,](#)

2010; [Narendra et al., 2010](#); [Rose et al., 2011](#)). PINK1 mutations C125G, I368N, and Q456X impact the ability of PINK1 to accumulate on the mitochondria in response to depolarization, while mutations A168P, H271Q, G309D, and W473X affect Parkin mitochondrial localization without interfering with PINK1 depolarization-induced accumulation ([Geisler et al., 2010b](#); [Narendra et al., 2010](#); [Ando et al., 2017](#); [Puschmann et al., 2017](#); [Sekine et al., 2019](#)). PD-associated Parkin mutations can prevent Parkin mitochondrial localization. However, C212Y, C289G, C418R, and C441R mutants tend to aggregate while K27N, R33Q, I44A, R46P, A46P, K211N, T240R, C253Y, Q331X, and G430D mutants remain soluble but are still unable to localize on the mitochondria ([Geisler et al., 2010a](#); [Narendra et al., 2010](#); [Rose et al., 2011](#); [Ham et al., 2016](#)). Parkin mutations K161N, A240R, R275W, and T415N have normal mitochondrial recruitment but are deficient in mitochondrial ubiquitination ([Geisler et al., 2010a](#); [Lee et al., 2010](#); [Narendra et al., 2010](#)). Interestingly, mutations with impaired Parkin recruitment tend to have a less severe mitophagy defect than mutations that completely inhibit Parkin translocation ([Narendra et al., 2010](#)). Although most PINK1 mutations cause PD through recessive inheritance, the inheritance of G411S appears to be dominant. When found in heterodimers, subtle structural changes at the dimer interface of G411S mutated PINK1 propagate

structural change to the WT PINK1 and interfere with its ubiquitin kinase activity (Puschmann et al., 2017).

Although less studied, additional genes have been linked to recessive PD with high confidence. They include *DJ-1*, *Fbxo7*, *PLA2G6*, *ATP13A2*, and *VPS13C*.

Mutations in each of these genes have been associated with altered mitophagy and defective mitochondrial degradation. Loss of function mutations in *DJ-1* are the third most common cause of recessive PD (Trempe and Fon, 2013). Analysis of rodents lacking DJ-1 revealed reduced AKT signaling and mislocalization of hexokinase 1 from mitochondria to the cytosol (Hauser et al., 2017). Both events appear to block mitochondrial localization of Parkin as well as subsequent ubiquitin phosphorylation (Hauser et al., 2017). *Fbxo7* PD-associated mutations have been linked to reduced Parkin translocation while *PLA2G6* PD-associated mutations have been associated with reduced protein levels of Parkin and BNIP3 (Burchell et al., 2013; Chiu et al., 2019). Deletion of the *C. elegans* ortholog of *ATP13A2* increases lysosomal pH and effectively reduces lysosomal degradative ability because lysosomal proteases require an acidic environment for maturation (Anand et al., 2020). Unlike the other PD associated mutations, modeling of PD-associated VPS13C loss of function appears to cause PINK1 and Parkin accumulation perhaps reflecting a functional downstream block of mitophagy (Lesage et al., 2016). Proteins in the VPS family are involved in vesicular transport

and VSP13C is specifically implicated in protein delivery to the lysosome (Lesage et al., 2016). Since VPS13C loss of function mutations are associated with PD, reduced lysosomal protein delivery could potentially alter lysosomal degradative ability and thus mitophagy (Blauwendraat et al., 2020).

SNCA, *GBA*, *VPS35*, and *LRRK2* mutations as well as *SNCA* gene duplication events are associated with autosomal dominant forms of PD and appear linked to reduced mitophagy, albeit with conflicting results. This has been clearly shown with *SNCA*, the gene that encodes α -synuclein, where increased PINK1/Parkin activity, reduced Parkin translocation, defective complex I activity, or defective targeting of mitochondria for autophagy have been implicated with the *SNCA*-PD association (Chinta et al., 2010; Chen et al., 2018; Shaltouki et al., 2018; Erustes et al., 2021). The pathogenic role of *GBA* and *VPS35* mutations appears to be more straightforward with mitochondrial priming defects caused by reduced mitochondrial localization of Parkin, NBR1, and BNIP3L in the case of *GBA* mutations, and defective lysosomal activity in the case of *VPS35* mutations (Li et al., 2019; Hanss et al., 2021). The case for *LRRK2*-associated mutations is quite complex. Some studies suggest that *LRRK2* mutations cause increased mitophagy as evidenced by increased mitophagosome formation, increased colocalization of mitochondria and lysosomes, and reduced mitochondrial levels (Yakhine-Diop et al., 2019, 2021). However, a larger body of evidence suggests

that mitophagy is actually decreased in LRRK2 mutants. This evidence includes impairment of specific mitophagy steps and direct observation of mitochondrial degradation by utilizing live imaging techniques ([Hsieh et al., 2016](#); [Bonello et al., 2019](#); [Korecka et al., 2019](#); [Liu et al., 2020](#); [Wauters et al., 2020](#); [Singh et al., 2021](#)). An inverse relationship between LRRK2 kinase activity and mitophagy has been suggested. Indeed, PD-associated LRRK2 mutations cause increased kinase activity and are associated with decreased mitophagy while genetic or pharmacological inhibition of LRRK2 kinase activity restores mitophagy ([Bonello et al., 2019](#); [Korecka et al., 2019](#); [Wauters et al., 2020](#)). However, there is no consensus on how LRRK2 activity mechanistically regulates mitophagy. Studies showing reduced mitophagy and normal autophagy suggest that the defect is in an early mitophagy-specific step ([Wauters et al., 2020](#); [Singh et al., 2021](#)). LRRK2 mutations have been proposed to hinder both Parkin mitochondrial recruitment and Miro removal ([Hsieh et al., 2016](#); [Bonello et al., 2019](#)). These two mechanistic features are likely linked as Miro is a target for Parkin mediated ubiquitination, and ubiquitinated Miro is degraded by the proteasome ([Birsa et al., 2014](#); [Springer and Macleod, 2016](#)). Miro removal halts mitochondrial motility and is thought to be a required step for mitophagy; both Miro removal and mitophagy are delayed in cells with LRRK2 mutations ([Birsa et al., 2014](#); [Hsieh et al., 2016](#)). The same delay in Miro removal and mitophagy was also observed in patient-derived fibroblast lines

with PINK1 and Parkin mutations as well as lines derived from sporadic PD patients, thus suggesting that delayed Miro removal could be a common mechanism for multiple causes of PD ([Liu et al., 2012](#); [Hsieh et al., 2016](#)). Decreased OPTN mitochondrial recruitment is also observed with LRRK2 mutations and –presumably– results from LRRK2-mediated RAB10 phosphorylation. RAB10 is normally found on depolarized mitochondria in close contact with OPTN, and this colocalization is decreased in LRRK2 mutant cells since LRRK2 activity increases levels of phosphorylated RAB10 ([Wauters et al., 2020](#)). Alternatively, some studies show an upregulation of initial mitophagy steps such as mitochondrial ubiquitination, p62 recruitment, and mitophagosome accumulation but have an overall decrease in mitochondrial degradation ([Liu et al., 2020](#)). This indicates a defect in mitophagy at a converging point with the general autophagy as mitophagosomes and autophagosomes are both degraded the same way. Evidence of reduced autophagic flux includes reduced formation of mature autophagosomes, and reduced lysosomal number, total area, and mean size ([Korecka et al., 2019](#); [Walter et al., 2019](#)).

Mitophagy is also impaired in cell lines derived from sporadic PD patients ([Hsieh et al., 2016](#); [Yakhine-Diop et al., 2019](#)). Interestingly, like patients with LRRK2 mutations, sporadic PD patients also have increased LRRK2 kinase activity ([Esteves et al., 2015](#)). This would then suggest that shared mechanisms could alter

mitophagy in both populations. Indeed, both have impaired Miro removal, and as a result, mitochondrial arrest and mitophagy are delayed ([Hsieh et al., 2016](#)). Direct assessment of over 70 patient-derived lines, 43 of which were from patients with sporadic PD, found defective Miro removal in 93% of all the lines ([Hsieh et al., 2019](#)). Large scale genetic studies have also helped connect sporadic PD to known mechanisms involved in monogenic PD ([Ivatt et al., 2014](#); [Billingsley et al., 2019](#)). For example, the identification of SREBF-1, a previously known risk locus for sporadic PD, in a screen for genes that regulate PINK1/Parkin mediated mitophagy highlights mitophagy dysfunction as a shared mechanistic link between autosomal recessive PD and at least some cases of sporadic PD ([Ivatt et al., 2014](#)).

Additionally, a GWAS study of sporadic PD patients identified 14 new genes causally associated with PD risk that were involved with various aspects of mitochondrial function including mitophagy, mitochondrial bioenergetics, and mitochondrial proteostasis ([Billingsley et al., 2019](#)). The data reported above provides strong support to the conclusion that mitochondrial dysfunction is a key aspect of PD pathophysiology and that it is implicated with both monogenic and sporadic forms of the disease. A schematic summary of mitophagy specific steps that are altered in PD are listed in [Figure 2](#).

Mitophagy dysfunction in Alzheimer's disease

AD is the most prevalent neurodegenerative disorder with over 55 million people living with this disease worldwide. Based on the average lifespan, it is estimated that almost 150 million individuals will develop AD by 2050 (Chowdhary et al., 2022). AD is also the sixth most common cause of death accounting for about 30 deaths per 100,000 in 2018 and 2019 (Kochanek et al., 2019; Rajan et al., 2021). The entorhinal cortex and hippocampus are among the first brain regions impacted by AD-associated degeneration, but eventually the disease becomes so widespread to affect both cortical and sub-cortical areas and cause a marked reduction in brain volume (Johnson et al., 2012). Symptoms initially include memory loss and language problems, but eventually basic bodily functions like walking and swallowing are impacted by the progressive neurodegeneration (Alzheimer's Association, 2021). Accumulation of amyloid-beta ($A\beta$) into extracellular amyloid (senile) plaques and hyperphosphorylated-Tau into intraneuronal neurofibrillary tangles (NFTs) help distinguish AD from other types of dementias that present with similar symptoms.

A large body of literature suggests that mitochondrial dysfunction is associated with the progression of AD. Mitochondria in AD patient brains have abnormal morphology, including reduced size, swollen shape and abnormal or reduced levels

of cristae ([Ye et al., 2015](#); [Fang et al., 2019](#)). Higher numbers of mitochondria have also been observed; however, this was only significant in AD brains with high NFT load suggesting that accumulation of mitochondria may occur later in disease progression ([Hu et al., 2016](#)). A summary of mitochondria alterations associated with AD are listed in [Table 1](#). The above changes in mitochondrial morphology are consistent with altered mitophagy flux and could either reflect increased mitophagy as damaged parts or damaged organelles need to separate from the larger mitochondrial network for mitophagy to occur, or could reflect decreased mitophagy as the accumulation of damaged mitochondria may be evidence of a degradation blockage. The observed structural changes are also associated with functional changes as hippocampal tissue from AD patients displays signs of energy deficit ([Fang et al., 2019](#)). Gene expression studies revealed reduced expression of autophagy- and mitophagy-associated genes in AD patient brains at the mRNA

(*ATG12, ATG5, BECN1, OPTN, ULK1, AMBRA1, BNIP3, BNIP3L, FUNDC1, VDAC1, VCP*) and protein (PINK1, BCL2L13, BNIP3L, p-TBK1, p-ULK1) level ([Martín-Maestro et al., 2017](#); [Fang et al., 2019](#)). Imaging studies looking at the colocalization of the mitochondrial marker TOMM20 and lysosomal marker LAMP2 indicate reduced mitophagy in AD patient brains ([Fang et al., 2019](#)). This finding coexists with an increase in autophagic vesicles, suggesting that the

progression of mitophagy, rather than the “marking” of mitochondria for mitophagic degradation, is affected ([Ye et al., 2015](#)). Interestingly, PINK1/Parkin tagging of mitochondria for degradation appears to be upregulated in the brain of AD patients as reflected by both higher total protein levels and higher association with mitochondria in late stage AD; this might potentially reflect an attempt to compensate for the dysfunctional or inefficient mitophagy ([Ye et al., 2015](#); [Martín-Maestro et al., 2016](#)). Consistently, pSer65-Ub, a specific marker of PINK1-Parkin pathway activity, is increased in AD brains ([Narendra, 2021](#)). Similar changes in mitochondrial morphology, ATP levels, and expression of autophagy- and mitophagy-related genes are observed in induced pluripotent stem cells (iPSCs) derived from familial and sporadic AD patients, thus, indicating the importance of mitochondrial dysfunction regardless of disease etiology ([Fang et al., 2019](#)).

Models for studying AD that mimic its characteristic accumulation of NFTs and amyloid plaques recapitulate the same dysfunctional mitochondria phenotypes observed in patient-derived samples. Mice with overexpression of human Tau (hTau) and *C. elegans* with phosphomimetic mutations in single-copy expression of hTau isoform 0N4R show similar mitochondrial changes, including increased numbers of mitochondria and more fragmented mitochondria ([Hu et al., 2016](#); [Guha et al., 2020](#)). The expression levels of transcription factors that regulate mitochondrial biogenesis, such as PGC-1 α , and TFAM, are unchanged in

both cellular and mouse models of hTau overexpression, supporting the argument that the observed accumulation of mitochondria results from a failure of mitophagy and not from the upregulation of the essential mitochondrial biogenesis machinery (Hu et al., 2016). Multiple studies have shown that Tau negatively regulates stress-induced mitophagy either by reducing or completely preventing mitophagy, depending on the model and stressor used (Hu et al., 2016; Cummins et al., 2019; Guha et al., 2020). Different models of Tau overexpression have found conflicting results regarding the impact of Tau overexpression on MMP at basal conditions and in response to stress, and have thus suggested different mechanisms for Tau-driven mitophagy interference. Tau overexpressing cells that exhibit reduced depolarization in response to CCCP also have increased Tau accumulation in the OMM, thus suggesting that Tau interferes with MMP through this mislocalization (Hu et al., 2016). The changes in MMP functionally impact mitophagy by decreasing the voltage-dependent mitochondrial localization of PINK1 and Parkin (Hu et al., 2016). Alternatively, Tau might affect mitophagy by binding and “trapping” Parkin in the cytosol (Cummins et al., 2019).

AD models based on APP (amyloid precursor protein) or A β overexpression, or A β exposure also show increasingly fragmented mitochondrial networks with higher total numbers of mitochondria and reduced mitochondrial length likely caused by a shift in mitochondrial fission/fusion dynamics (Ye et al., 2015; Hu et al.,

2016; Martín-Maestro et al., 2017; Castellazzi et al., 2019). These mitochondria are dysfunctional, exhibiting a reduced membrane potential, decreased respiration rates, and increased release of reactive oxygen species (Ye et al., 2015; Han et al., 2017, 2020; Sorrentino et al., 2017). Expression changes at the mRNA and protein levels suggest that decreased mitochondrial biogenesis and reduced mitophagy both play a role in the accumulation of dysfunctional mitochondria (Manczak et al., 2018; Reddy et al., 2018). Reduced retrograde transport of axonal mitophagosomes may contribute to the mitophagy deficit observed in the brains of transgenic mice overexpressing mutant versions of human APP associated with hereditary/familial forms of AD; mitophagosomes accumulate in presynaptic terminals separated from lysosomes that are concentrated in the soma of neurons (Han et al., 2020). Changes in axonal transport dynamics that would support increased overall movement toward the soma, increased retrograde transport and decreased anterograde transport, have also been described. The accompanying accumulation of somal mitophagosomes indicates impaired lysosomal degradation rather than defects in axonal transport (Ye et al., 2015). It is worth noting that the above mentioned studies observed axonal transport on different time scales (100 s vs. 9 min) which could contribute to whether the accumulation of mitophagosomes was respectively, observed in the axon or in the soma (Ye et al., 2015; Han et al., 2020). Expression of DISC1, which was recently discovered to function as a

mitophagy receptor, is decreased in post mortem brains of AD patients and in symptomatic APP/PS1 mice indicating a different type of priming failure that is functionally relevant in AD. Indeed, rescuing DISC1 expression reduces A β plaque accumulation and synaptic loss, and improves behavioral performance on cognitive tests in APP/PS1 mice (Wang Z.-T. et al., 2019). A schematic summary of mitophagy specific steps that are altered in AD are listed in Figure 2.

Mitophagy dysfunction in Huntington's disease

Huntington's disease (HD) is a rare, monogenic, autosomal dominant neurodegenerative disorder affecting an estimated 5–10 people per 100,000 that is caused by expansion of the polyglutamine (polyQ) region in the huntingtin protein (HTT) (Labbadia and Morimoto, 2013). Trinucleotide repeats of 36 or more are pathogenic with repeat lengths 40 or greater causing full penetrance, and repeat lengths of 36–39 causing incomplete penetrance. People with fewer than 35 repeats usually do not develop HD, although carrying 27–35 repeats may lead to some HD-like symptoms, presumably caused by somatic expansion (Labbadia and Morimoto, 2013; Stoker et al., 2022). Mutant HTT (mHTT) forms aggregates and exhibits pathogenicity through gain-of-function rather than loss-of-function effects (Labbadia and Morimoto, 2013; Stoker et al., 2022). Degeneration is widespread but particularly evident in the GABAergic neurons of the striatum (Stoker et al.,

2022). Patients typically experience neuropsychiatric and cognitive symptoms such as apathy, personality changes, and deficits in executive functioning prior to the characteristic choreiform movements which are used to define disease onset (Stoker et al., 2022).

Fibroblasts derived from HD patients exhibit more fragmented mitochondrial morphology and are functionally impaired, with increased ROS generation, decreased MMP, and decreased ATP production (Hwang et al., 2015). Similarly, HD patient iPSC-derived neurons have shorter mitochondria and also exhibit a reduced MMP (Guo et al., 2016). Cellular and animal models of HD recapitulate both the morphological and functional mitochondrial phenotypes observed in the patient derived cell lines in regards to changes in mitochondrial length and fragmentation, ROS and ATP production, and MMP (Hwang et al., 2015; Khalil et al., 2015; Guo et al., 2016; Franco-Iborra et al., 2021). Additional aspects of mitochondrial morphology found altered in HD-like models include functional changes- decreased mitochondrial respiration- and morphological changes- rounder mitochondria with a spheroid shape, abnormal cristae, and an increase in the overall number of mitochondria (Hwang et al., 2015; Khalil et al., 2015; Franco-Iborra et al., 2021). A summary of mitochondria alterations associated with HD are listed in Table 1.

Unchanged mitochondrial protein ubiquitination and decreased LC3 recruitment to the mitochondria suggest that, mechanistically, mitophagy dysfunction occurs downstream of PINK1/Parkin-mediated tagging to damaged mitochondria but upstream of mitophagosome formation ([Khalil et al., 2015](#); [Franco-Iborra et al., 2021](#)). Though PINK1 expression is unchanged in HTT mutants, increased expression of PINK1 is capable of rescuing both mitophagy defects and mitochondrial dysfunction ([Khalil et al., 2015](#)). PINK1-driven rescue is dependent on Parkin functions, supporting the conclusion that rescue occurs by increasing tagging of damaged mitochondria to compensate for the downstream deficit in mitophagy ([Khalil et al., 2015](#)). Targeting a microRNA, miR-302, that is downregulated in cell models of HD also has a rescue effect, improving cell viability and reducing the number and size of mHTT aggregates ([Chang et al., 2021](#)). Upregulating miR-302 increases Sirt1 protein levels and AMPK phosphorylation; both events have been shown to increase expression of ATGs ([Chang et al., 2021](#)). Thus increasing targeting of damaged mitochondria and increasing the expression of autophagy machinery have both been shown to rescue the mitophagy deficit exhibited by HD models.

Mechanistic studies revealed multiple potential mechanisms for how mHTT can disrupt mitophagy. Specifically, altered engagement of mitophagy receptors (OPTN and NDP52), as well as the autophagy induction machinery (ULK1 and

BECN1) have been proposed ([Franco-Iborra et al., 2021](#)). HTT normally interacts with OPTN and NDP52, but this interaction is respectively, reduced or abolished between mHTT and OPTN or NDP52 ([Franco-Iborra et al., 2021](#)). Consequently, LC3 interaction with the receptors is also reduced indicating improper recognition of mitochondria for degradation ([Franco-Iborra et al., 2021](#)). HTT and MTORC1 normally compete for ULK1 binding: ULK1 is inactive when bound to MTORC1 and active when bound to HTT ([Franco-Iborra et al., 2021](#)). Cells expressing mHTT exhibit more ULK1 binding with MTORC1 and less ULK1 binding with mHTT, which functionally reduces ULK1 activation as evidenced by the increased levels of phosphorylated ULK1 at Ser757 ([Franco-Iborra et al., 2021](#)). HD cell models also show reduced formation of the PtdIns3K complex. This could be due to reduced ULK1 activity as ULK1 phosphorylates and activates the PtdIns3K complex member BECN1. Another alternative or complementary explanation relates to BECN1's ability to bind expanded polyQ repeats. Normally, BECN1 binding to the polyQ repeat in ATXN3 prevents its degradation. The presence of mHTT could competitively interfere with BECN1-ATXN3 binding and lead to increased degradation of BECN1 ([Franco-Iborra et al., 2021](#)). Defects in mitophagy might also occur further downstream. Indeed, increased mitophagosome accumulation along the axon and decreased numbers of acidified autophagosomes closer to the cell body have been described ([Wong and Holzbaur,](#)

2014b). Alternatively, there is evidence that excessive mitophagy might result from VCP binding mHTT in HD models (Guo et al., 2016). Normally VCP is necessary for the proteasomal degradation of Mfn1/2 after ubiquitination by Parkin, which blocks mitochondrial fusion as means to alleviate depolarization (Tanaka et al., 2010). However, VCP could also stimulate mitophagy by binding LC3 through proposed LIR domains (Guo et al., 2016). A schematic summary of mitophagy specific steps that are altered in HD are listed in Figure 2.

Chapter2

Mitophagy dysfunction in amyotrophic lateral sclerosis

ALS is a neurodegenerative disease that impacts both the brain and the spinal cord (Shatunov and Al-Chalabi, 2021; Siddique and Siddique, 2021). There are approximately 1.75–3 new cases per 100,000 persons every year (Masrori and Van Damme, 2020). Patients are typically in their mid-fifties to mid-sixties when early

symptoms manifest (Siddique and Siddique, 2021). Most patients initially experience asymmetric and focal weakness in distal limb muscles, more commonly in the dominant hand or tibial muscles (Masrori and Van Damme, 2020; Siddique and Siddique, 2021). The remaining one-third of ALS patients initially present bulbar muscle weakness which results in dysarthria (difficulty speaking) and/or dysphagia (difficulty swallowing) (Masrori and Van Damme, 2020; Siddique and Siddique, 2021). As the disease progresses, muscle weakness and atrophy increasingly spread to adjacent regions and can eventually result in paralysis (Masrori and Van Damme, 2020; Shatunov and Al-Chalabi, 2021; Siddique and Siddique, 2021). Median survival is 3–5 years after symptoms onset, and death is frequently caused by respiratory muscle failure (Masrori and Van Damme, 2020; Siddique and Siddique, 2021).

Though ALS was traditionally characterized as familial or sporadic, convention is switching to classifying ALS as “genetic ALS” or “ALS of unknown cause” because genetic testing has revealed that 14% of patients with no family history have mutations in ALS genes and 20% of patients with family history lack mutations in ALS genes (Shatunov and Al-Chalabi, 2021; Siddique and Siddique, 2021). There are currently 20–30 well-established genes associated with increased ALS risk. The top five, by prevalence, are *C9ORF72*, *SOD1*, *FUS*, *TARDBP*, and *TBK1* (Masrori and Van Damme, 2020; Shatunov and Al-Chalabi,

2021; [Siddique and Siddique, 2021](#)). In 95–97% of ALS patients, TDP-43, the protein product of *TARDBP*, is found mislocalized from the nucleus into cytoplasmic aggregates ([Masrori and Van Damme, 2020](#); [Shatunov and Al-Chalabi, 2021](#)). TDP-43 pathology has been found in all types of ALS patients excluding patients with *FUS* and *SOD1* mutations where instead, aggregates of the respective mutated proteins accumulate ([Masrori and Van Damme, 2020](#); [Shatunov and Al-Chalabi, 2021](#)). Studies utilizing post-mortem brain tissue from patients with TDP-43 proteinopathy and fibroblast lines derived from patients harboring ALS-associated mutations in *TARDBP* or *C9ORF72* demonstrate mitochondrial abnormalities ([Onesto et al., 2016](#); [Wang P. et al., 2019](#)). Mitochondria appear rounder, more fragmented, and display swollen or partial to complete loss of cristae ([Onesto et al., 2016](#); [Wang P. et al., 2019](#)). Morphological changes in mitochondrial fragmentation, shape, and cristae structure are reproduced in ALS cellular and animal models ([Xu et al., 2010](#); [Wang et al., 2013](#); [Wang P. et al., 2019](#); [Evans and Holzbaur, 2020a](#); [Jun et al., 2020](#)). Patient-derived fibroblasts also provide evidence that mitochondrial function is altered in ALS in addition to morphology ([Onesto et al., 2016](#)). Some of the observed functional changes appear to be dependent on the specific ALS-associated mutation. Cells harboring *TARDBP* and *C9ORF72* mutations showed significant changes in fission/fusion dynamics, membrane polarization, and mitochondrial mass, but the

directionality of the changes was different between the two models ([Onesto et al., 2016](#)). Mirroring this, TDP-43 ALS models generally point to increased mitochondrial fission and decreased fusion ([Xu et al., 2010](#); [Wang et al., 2013](#)). Though the reverse has also been observed, increased fission and decreased fusion is consistent with the previously mentioned increased mitochondrial fragmentation, strengthening the case for these changes ([Wang et al., 2013](#); [Onesto et al., 2016](#); [Davis et al., 2018](#); [Wang P. et al., 2019](#); [Jun et al., 2020](#)). Patients with *C9ORF72* hexanucleotide expansion appear to exhibit a unique functional mitochondrial phenotype: TDP-43, SOD1, and OPTN based ALS models all have depolarized MMP whereas MMP is hyperpolarized in *C9ORF72* models ([Hong et al., 2012](#); [Wang et al., 2013](#); [Xie et al., 2015](#); [Onesto et al., 2016](#); [Wang P. et al., 2019](#); [Evans and Holzbaur, 2020a](#); [Jun et al., 2020](#)). *C9ORF72* models are also unique in having increased ATP content and maximum oxygen consumption rate while other ALS models exhibit decreased ATP synthesis and maximum oxygen consumption ([Mattiuzzi et al., 2002](#); [Onesto et al., 2016](#); [Wang P. et al., 2019](#)). However, these differences could potentially be explained by increased biogenesis in *C9ORF72* models, as evidenced by increased PGC1- α , in attempt to compensate for dysfunctional mitochondria ([Onesto et al., 2016](#)). Increased levels of mitochondrial ROS across models suggests a shared failure of mitochondrial function ([Mattiuzzi et al., 2002](#); [Hong et al., 2012](#); [Wang et al., 2013](#); [Xie et al.,](#)

2015; [Onesto et al., 2016](#); [Wang P. et al., 2019](#); [Tak et al., 2020](#)). A summary of mitochondria alterations associated with ALS are listed in [Table 1](#).

TDP-43 colocalizes with mitochondrial markers in spinal cord and brain tissue from ALS patients and in patient-derived fibroblast lines containing *TARDBP* mutations, suggesting a potential mechanism by which TDP-43 could affect mitochondrial function ([Wang et al., 2016](#); [Wang P. et al., 2019](#)). This finding has been replicated in primary mouse cell lines and standard cell lines that exogenously express human TDP-43 (hTDP-43) harboring ALS-associated mutations as well as C-terminal TDP-43 fragments ([Hong et al., 2012](#); [Wang et al., 2013](#); [Wang P. et al., 2019](#); [Jun et al., 2020](#)). Importantly, the C-terminal fragments resulting from proteolytic cleavage of TDP-43 are found in TDP-43 cytoplasmic inclusions, while exogenous expression of hTDP-43 in mice results in ALS-like motor phenotypes including tremors, difficulty walking, and abnormal hindlimb clasping in addition to cytoplasmic TDP-43 aggregates in spinal cord motor neurons ([Shan et al., 2010](#); [Xu et al., 2010](#); [Jun et al., 2020](#)). Furthermore, preventing mitochondrial TDP-43 localization through deletion of the M1 region was able to normalize mitochondrial length, membrane potential, oxygen consumption rate, and ATP synthesis in cells overexpressing WT or mutant TDP-43, thus providing evidence that mitochondrial localization of TDP-43 contributes to mitochondrial dysfunction ([Wang et al., 2016](#)). Exogenous expression of TDP-

43 or C-terminal TDP-43 fragments also appeared to enhance mitophagy as evidenced by increased Parkin recruitment to mitochondria and increased levels of LC3-II ([Hong et al., 2012](#); [Davis et al., 2018](#); [Jun et al., 2020](#)). However, it is unclear if this increase results in increased degradation of mitochondria since TDP-43 expression also increases the percentage of stationary mitochondria in axons and neurites ([Cai et al., 2012](#); [Wang et al., 2013](#); [Evans and Holzbaur, 2020a](#)).

Mutations to *SOD1* are the second most common cause of genetic ALS and have been the subject of much research as *SOD1* was the first genetic cause of ALS discovered ([Rosen et al., 1993](#); [Masrori and Van Damme, 2020](#); [Shatunov and Al-Chalabi, 2021](#)). Mutations to *SOD1* seem to impact mitophagy in addition to mitochondrial morphology and functionality, as discussed earlier, though the specific mechanistic components (and consequences) are not as clear. Evidence supporting increased mitophagy resulting from ALS-associated *SOD1* mutations includes increased p62 recruitment to the mitochondria suggesting increased mitochondrial priming, decreased levels of mitochondrial proteins suggesting lower levels of mitochondria, and, most convincingly, increased mitophagy as demonstrated by the mt-Keima reporter in *SOD1* mutant mice ([Palomo et al., 2018](#)). Other studies have found that *SOD1* mutant motor neuron cell bodies and presynaptic terminals at the neuromuscular junctions (NMJs), respectively, have increased mitophagosome formation or no significant changes from WT which

appears consistent with increased mitophagy in some regions of the cell (Rogers et al., 2017). However, because the number of damaged mitochondria is greater in *SOD1* mutant presynaptic NMJ terminals than in WT, and the number of damaged mitochondria increases faster than mitophagosomes in *SOD1* mutant motor neuron cell bodies, a mitophagy defect is manifest (Rogers et al., 2017). Additional evidence pointing to a mitophagy defect in *SOD1* mutant models includes reduced levels of mitophagy proteins BNIP3, PINK1, and Parkin, fewer mitophagosomes, higher levels of mitochondria after stimulation with the mitophagy inducer CCCP, accumulation of p62-associated mitochondria, and abnormal degradative vesicles containing mitochondria (Xie et al., 2015; Rogers et al., 2017; Tak et al., 2020). One potential explanation for the observed mutant *SOD1*-associated mitophagy defects is its ability to interact with mitophagy receptor OPTN leading to the sequestration of OPTN to *SOD1* aggregates, which would hinder the recognition of damaged mitochondria (Tak et al., 2020). Mutant *SOD1* has also been shown to interact with retrograde trafficking protein dynein and to impair the interaction between dynein and endosomal adaptor trafficking protein Snapin (Xie et al., 2015). As a result, live imaging indicates hindered maturation of endocytic vesicles and autophagosomes, and reduced levels of mature lysosomes as this process requires axonal transport in neurons (Xie et al., 2015). Overexpression of Snapin restores the Snapin dynein interaction and

rescues the lysosomal phenotype seen in *SOD1* mutants in addition to restoring mitochondrial morphology and functionality, and ameliorating the ALS-like symptoms observed in the mouse ([Xie et al., 2015](#)).

ALS-associated mutations in *TBK1* and the functionally related protein OPTN have clearly established roles in mitophagy with OPTN recognizing damaged mitochondria through its ubiquitin-binding domain, and TBK1 phosphorylating OPTN and increasing the ubiquitin-binding ability of OPTN ([Wong and Holzbaur, 2014a](#); [Moore and Holzbaur, 2016](#); [Bader and Winklhofer, 2020](#); [Evans and Holzbaur, 2020b](#); [Montava-Garriga and Ganley, 2020](#); [Wang L. et al., 2020](#)). As such, it is not difficult to imagine how mutations to these proteins have the potential to alter mitophagy. Two ALS-associated mutations in *OPTN* have been found to likely affect its ubiquitin-binding ability either indirectly through changes to the dimeric structure of OPTN or directly through mutations in the ubiquitin-binding domain ([Li et al., 2018](#)). The latter has been shown to impact mitophagy resulting in decreased OPTN recruitment to damaged mitochondria, slower mitophagosome formation, and increased mitochondrial levels after mitophagy stimulation ([Wong and Holzbaur, 2014a](#); [Moore and Holzbaur, 2016](#); [Evans and Holzbaur, 2020a](#)). Another ALS-associated *OPTN* mutation, which results in a premature stop that eliminates the ubiquitin-binding domain, also has impaired mitophagosome formation indicating the importance of OPTN as a mitophagy

receptor ([Maruyama et al., 2010](#); [Moore and Holzbaur, 2016](#)). Multiple patient mutations in *TBK1* have reduced or completely abolished OPTN binding activity and expectedly, given the role of TBK1 in enhancing OPTN function, inhibit mitophagosome formation ([Freischmidt et al., 2015](#); [Moore and Holzbaur, 2016](#)).

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Chapter3

In 1895, Hens Gierke proposed the idea of a homogeneous and amorphous ground substance that embeds neuroglia and forms the structural architecture of the brain ([Celio, 1999](#)). Soon after, in 1898 Camillo Golgi described a pericellular coating around specific neurons in his seminal study on the eponymous Golgi complex ([Celio et al., 1998](#); [Celio, 1999](#)). After nearly a century of relative obscurity, these structures unambiguously established themselves as different forms of neuroglial-

embedding extracellular matrix (ECM) known as interstitial matrix and perineuronal nets (PNNs). Brain ECM is rich in hyaluronan (HA), chondroitin sulfate proteoglycans (CSPGs), and glycoproteins, with a minor proportion of fibrous proteins. Together in conjunction with water, ions, and secreted molecules, ECM creates a functionally dynamic extracellular milieu that provides structural support and effectuates diverse neuromodulatory functions (Hrabetova et al., 2018; Fawcett et al., 2019).

A large fraction of ECM is homogeneous and amorphous; however, several morphologically distinct forms are distributed throughout the brain (Fawcett et al., 2019; Patel et al., 2019; Chaunsali et al., 2021). A thin sheet-like condensation of ECM molecules on the pial surface and around parenchymal vasculature forms basement membranes (BMs) which carries out structural, signaling, and barrier functions. Another phenotypic specialization of ECM is PNN, which is a lattice-like condensation predominantly juxtaposing the soma, dendrites, and axon initial segment (AIS). A vast majority of PNN-expressing neurons are fast-spiking parvalbumin (PV)-expressing GABAergic neurons; however, several non-PV neurons also express PNNs (Lensjø et al., 2017a; Patel et al., 2019; Chaunsali et al., 2021). Brain ECM, including PNNs, is spatiotemporally malleable and maintains a characteristic composition and structural organization at different stages of pre and postnatal development, adulthood, aging, and central nervous

system (CNS) pathologies. The key advantage of the malleability appears to be a functional versatility, owing to which the ECM and PNNs perform diverse functions at specific stages of life. Since functional versatility is predominantly determined by spatiotemporal dynamics, the central question arises; what regulates the ECM and PNN dynamics and thereby critically determines their functions?

Recent studies suggest that the structural organization of ECM and PNNs, and therefore their functions, are regulated by intrinsic mechanisms- driven by neurons- as well as extrinsic- driven primarily by glial cells (Wiese et al., 2012; Rowlands et al., 2018; Crapser et al., 2021; Ribot et al., 2021). CNS glia, including astrocytes, oligodendrocytes, and microglia are capable of producing ECM and PNN components and are significant sources of ECM during development and adulthood (Wiese et al., 2012; Song and Dityatev, 2017). In addition, astrocytes excessively produce ECM molecules under several CNS pathologies, effectuating both protective and detrimental outcomes (Fitch and Silver, 2008; Kim et al., 2016, 2017; George and Geller, 2018). Besides producing ECM molecules of structural and signaling utility, astrocytes release an array of diverse matrix-remodeling proteases and their inhibitors to tightly control the structural integrity of PNNs and ECM (Fitch and Silver, 2008; Patel et al., 2019; Chaunsali et al., 2021).

While astrocytes are mainly engaged with the synthesis and release of ECM and their proteolytic enzymes, it is microglia that contribute significantly to the continuous elimination of the ECM molecules due to their characteristic phagocytic property. Normally, the homeostatic states of ECM and PNNs are maintained by a constitutive expression of ECM and proteases by neurons and astrocytes, as well as clearance by microglia. However, as seen in several recent studies on epilepsy, Alzheimer's disease (AD), Huntington's disease (HD), neuropathic pain, etc., dysfunctional microglia leads to abnormal clearance or accumulation of the ECM and PNNs contributing to the pathology (Tewari et al., 2018; Patel et al., 2019; Crapser et al., 2020b,2021; Chaunsali et al., 2021; Carceller et al., 2022; Tansley et al., 2022).

In this review, we discuss the classic roles of and recent advances in the functions of ECM and PNNs, followed by the role of glial cells in ECM and PNN remodeling in healthy brain and pathologies. These roles suggest a pivotal contribution of glial cells to this remodeling process and thus encourage a discussion on a glia-centric approach to treatment strategies.

Structure and functions of extracellular matrix and perineuronal nets in the central nervous system

Extracellular matrix is present in all tissues of the body as a structural framework of amorphous and diffused interstitial matrix; however, brain ECM is unique in its composition and organization. From a composition point of view, a major fraction of the brain ECM consists of glycosaminoglycans (GAGs), proteoglycans, and glycoproteins, with a negligible fraction of fibrous proteins which is contrary to the fibrous protein-rich ECM in a majority of other tissues (McRae and Porter, 2012). Another key feature of the brain ECM is its structural organization into distinct forms such as thin sheets of BMs and highly condensed pericellular coats of PNNs.

Basement membranes

Basement membrane is an organized ECM assembly in the form of thin sheets that surround the pial surface (meningeal BM) and brain vasculature (vascular BM) (Thomsen et al., 2017). Similar to other forms of ECM, the BMs also show a spatiotemporally dynamic composition which determines their functions at different stages of life. By and large, collagen IV, laminins (1–5), nidogens (1 and 2), and heparin sulfate proteoglycans (HSPGs) (perlecan and agrin) are the most static components (Thomsen et al., 2017). On the other hand, insoluble fibronectin,

fibulins, thrombospondins (TSPs), and secreted protein acidic and rich in cysteine (SPARC) are more dynamic and are expressed at specific developmental and pathophysiological states ([Thomsen et al., 2017](#)). Besides serving as a major route *via* which fluids and soluble molecules enter and leave the brain, BMs provide structural support by acting as an adhesive substrate for cells to anchor to and mediate signal transduction *via* integrin and other transmembrane matrix receptors ([Baeten and Akassoglou, 2011](#)). Meningeal BM is critical for brain development and the absence of the BM or its constituents causes abnormal brain development ([Halfter et al., 2002](#)). The vascular BM plays a critical role in maintaining the blood-brain barrier (BBB), as evidenced by BBB disruption and cerebrovascular defects in the absence of BM components such as laminins ([Yao et al., 2014](#)) and collagens ([Engelhardt, 2003](#); [Jeanne et al., 2015](#)). In several CNS disorders, predominantly in stroke and traumatic brain injury (TBI), BBB disruption is associated with an altered BM, causing an infiltration of otherwise impermeable serum components and immune cells to trigger inflammation and subsequently neuroglial dysfunctions ([Thomsen et al., 2017](#)). Extravasation of blood proteins fibrinogen and albumin trigger molecular changes in astrocytes, transforming them into their reactive state which in turn further remodels the ECM and forms glial scars ([Kim et al., 2016, 2017](#)) (discussed later).

Interstitial matrix

Historically, the idea of ECM was pioneered as a neuroglia-embedding structural framework of a diffused, amorphous, and ubiquitously distributed ground substance in the extracellular space (ECS) ([Celio, 1999](#)). This form is now known as interstitial matrix and constitutes the highest fraction of brain ECM. Interstitial matrix fills nearly the entire ECS and embeds other phenotypes of ECM such as perineuronal, perisynaptic, and perinodal matrices ([Engelhardt, 2003](#); [Lau et al., 2013](#); [Fawcett et al., 2019](#)). The meshwork of the interstitial matrix consists of hyaluronan, proteoglycans, tenascins, link proteins, glycoproteins such as laminins and fibronectin, and a relatively small fraction of fibrous proteins such as collagens and elastin ([Rauch, 2007](#); [Lau et al., 2013](#); [Lei et al., 2017](#)). Several transmembrane and membrane-coupled proteins and receptors including CD44, receptor for hyaluronan-mediated motility (RHAMM), Stabilin-2, TNFIP6, SHAP, TLR-2, and TLR-4 are connected directly with the hyaluronan to anchor and stabilize the ECM ([Jiang et al., 2011](#)). Similarly, chondroitin sulfate binds to several transmembrane receptors including RPTP σ , LAR, RPTP δ , and Nogo receptors as well as adhesion molecules including NCAM and integrins ([Yu et al., 2018](#)). The interstitial matrix harbors ions, secreted molecules such as growth factors and neuromodulatory agents, and most importantly, provides a high

hydration capacity to maintain ECS volume and thereby normal brain activity (Perkins et al., 2017; Hrabetova et al., 2018).

A large fraction of diffused interstitial matrix coats the synapses, forming a perisynaptic matrix, and is involved in synaptogenesis and plasticity often under the regulation of matrix remodeling enzymes (Orlando et al., 2012; Korotchenko et al., 2014; Fawcett et al., 2019). Depletion of perisynaptic HA affects synaptic potentiation by altering the lateral mobility of AMPARs (Frischknecht et al., 2009) as well as the activity of L-type voltage-dependent calcium channels (L-VDCCs) at synaptic terminals (Kochlamazashvili et al., 2010). Similarly, Tenascin-C (Tn-C) deficiency impairs synaptic plasticity by altering L-VDCCs signaling, however, Tenascin-R (Tn-R) deficiency, which is expressed around perisomatic synapses, alters NMDAR-dependent LTP by reducing the perisomatic inhibition (Evers et al., 2002; Hayani et al., 2018). More recently, Tn-R appears to be recycled at the active synapse in an activity-dependent manner influencing the synaptic structure (Dankovich et al., 2021). These studies suggest a pivotal role of interstitial matrix molecules in effectuating the dynamic changes at synapses.

Besides PNNs, few other specialized phenotypes of the ECM are embedded largely within the diffused interstitial matrix. For example, perinodal ECM is a condensed form of ECM around the nodes of Ranvier (Bekku and Oohashi, 2019) consisting

of Tn-R, brevican, versican, phosphacan, Bral1, and neurocan ([Susuki et al., 2013](#)). Tn-R plays an essential role in axonal functions presumably by acting as an ion diffusion barrier ([Bekku and Oohashi, 2019](#)) as evidenced by decreased axonal conduction velocity in the optic nerve in Tn-R deficient condition ([Weber et al., 1999](#)). Axonal coats are another phenotypic specialization of ECM which are rich in CSPGs, including aggrecan and brevican; however their functional relevance is elusive ([Morawski et al., 2012](#); [Jäger et al., 2013](#)). Recent studies support the presence of brevican and NG2 expressing axonal coats surrounding myelinated axons in human brains and are suggested to aid axonal properties ([Pantazopoulos et al., 2022](#)).

Perineuronal nets

Historically, PNNs have been the most intriguing yet enigmatic ECM structures. PNNs are widely expressed in several brain regions including the cerebral cortex, amygdala, striatum, and hippocampus ([Morikawa et al., 2017](#); [van't Spijker and Kwok, 2017](#); [Ulbrich et al., 2021](#)) as well as in the spinal cord ([Irvine and Kwok, 2018](#)) of rodents and humans ([Chaunsali et al., 2021](#); [Carceller et al., 2022](#)). PNNs are predominantly present on the fast-spiking PV interneurons; however, a small population of other inhibitory and excitatory neurons in brain and spinal cord express PNNs ([Irvine and Kwok, 2018](#); [Chaunsali et al., 2021](#)). The typical lattice

of PNN is a ternary complex of hyaluronan, link proteins (HAPLNs), proteoglycans of the lectican family or CSPGs including aggrecan, brevican, versican, and neurocan, and tenascin glycoproteins (Tn-C, Tn-R). PNNs can be visualized by fluorescently labeled antibodies that bind to the core proteins or by lectins such as Wisteria floribunda agglutinin (WFA) that bind the GAGs sidechains ([Fawcett et al., 2019](#); [Tewari and Sontheimer, 2019](#); [Figure 1A](#)). The cavities of the PNN lattice on the soma, AIS, and dendrites house both excitatory and inhibitory synaptic terminals ([Fawcett et al., 2019](#); [Carceller et al., 2020](#)).

The key role of anchoring the extracellular components of PNNs to the cell membrane is performed by HA-producing transmembrane enzymes, hyaluronic acid synthase (HAS 1-3) ([Kwok et al., 2011](#)). HAS-associated long chains of HA are connected directly to the link proteins (HAPLN), which in turn bind to the CSPG core proteins. The core proteins of lecticans also form a backbone to which numerous side chains of sulfated GAGs are attached. Lecticans are cross-linked by Tn-R to further secure the assembly ([Figure 1B](#)) and a loss of aggrecan crosslinking by Tn-R impairs the PNN assembly around dendrites ([Morawski et al., 2014](#)). Despite being a multimolecular assembly, WFA-labeled PNNs remain minimally distorted in the absence of single or multiple PNN components including HA ([Arranz et al., 2014](#)), neurocan ([Zhou et al., 2001](#)), brevican ([Brakebusch et al., 2002](#)), Tn-C ([Irintchev et al., 2005](#); [Gottschling et al., 2019](#)),

Tn-R ([Brückner et al., 2000](#); [Gottschling et al., 2019](#)), and link proteins ([Carulli et al., 2010](#)). However, as the one indispensable component of the PNN, aggrecan deficiency leads to the absence of PNNs ([Giamanco et al., 2010](#); [Rowlands et al., 2018](#)) (See review [Carceller et al., 2022](#)).

In different regions of the developing mouse brain, traces of PNN appear at different ages and gradually achieve fully condensed arborization in several weeks. For example, immature PNNs can be identified in brainstem by postnatal day 4 ([Brückner et al., 2000](#)); however in the cerebral cortex and amygdala by postnatal days 14 and 21, respectively ([Brückner et al., 2000](#); [Horii-Hayashi et al., 2015](#)). In humans, PNNs appear near 8 weeks in medial prefrontal cortex and mature around 8 years of age ([Rogers et al., 2018](#)). By and large, this developmental trajectory of PNN formation coincides with the critical periods of heightened neuroplasticity, in which neuronal circuits are highly responsive to sensory inputs and brain connections are established and strengthened in an activity-dependent manner ([Pizzorusso et al., 2002](#)). Sensory deprivation within the critical period permanently disrupts the normal development of the brain circuits; however, sensory deprivation outside the critical period or in adults does not affect neuronal circuits and brain functioning ([Hubel and Wiesel, 1965, 1970](#); [Reh et al., 2020](#)). Pioneering studies have shown that preventing PNN formation in the developing brain prolongs the critical period of plasticity, and that disruption of PNNs outside

the critical period using a bacterial-derived enzyme Chondroitinase ABC (ChABC) reinstates neuroplasticity similar to that of the critical period, suggesting PNNs as the primary regulator of the critical period plasticity (Pizzorusso et al., 2002; Wang and Fawcett, 2012; Rowlands et al., 2018; Fawcett et al., 2019).

Despite a progressive condensation of CSPGs into PNNs in the developing brain, the total CSPG content remains largely unchanged- which complicates the question of mechanisms whereby PNN CSPGs are inhibitory to neuroplasticity (Miyata et al., 2012). Intriguingly, the plasticity-permissive nature of the developing brain is attributed to a characteristic sulfation pattern of the CSPGs. The developing brain exhibiting immature PNNs and high neuroplasticity possesses a higher C6S proportion than C4S to maintain a low C4S/C6S ratio. Over the developmental period, the ratio changes to a higher C4S/C6S, which not only promotes PNN maturation but also suppresses neuroplasticity (Miyata et al., 2012; Miyata and Kitagawa, 2016; Foscarin et al., 2017). Nevertheless, the downstream cellular and molecular mechanism behind the inhibitory nature of a higher C4S/C6S ratio remains elusive.

Developmental formation of PNNs is activity-dependent, and several brain regions including barrel cortex, thalamus, visual cortex, and vocal center in songbirds show underdeveloped PNNs if deprived of activity, suggesting a high malleability

of PNNs ([Guimarães et al., 1990](#); [Lander et al., 1997](#); [Pizzorusso et al., 2002](#); [McRae et al., 2007](#); [Nakamura et al., 2009](#)). Although PNNs in the mature CNS appear to be largely stable in a normal physiological state, emerging evidence suggests bidirectional changes in the structure and numerical density of PNNs on a cyclic basis as well as under specific conditions such as drug addiction, maternal hormone fluctuations, and chronic pain ([Lasek et al., 2018](#); [Pantazopoulos et al., 2020](#); [Uriarte et al., 2020](#); [Harkness et al., 2021](#); [Mascio et al., 2022](#)).

Functions of perineuronal nets

Chondroitin sulfate proteoglycans are critical constituents of the PNNs and several signaling functions of CSPGs are independent of their phenotypic appearance as PNNs or interstitial matrix as evidenced in the following studies. In the extensively studied visual system, a high density of CSPGs repels the growing retinal axons to navigate them to their target areas in the developing brain. Conversely, depleting CSPGs with ChABC is disruptive to the axonal guidance and misleads the axons to non-target areas ([Brittis et al., 1992](#); [Laabs et al., 2005](#)). Since axonal growth and guidance is a developmental phenomenon, the inhibitory role of CSPGs appears extraneous in adult CNS physiology. However, in CNS injury and trauma, the damaged axons fail to regenerate due to the CSPG-rich glial scar at the injury site

and ChABC-mediated removal of CSPGs improves the repair and regeneration and to a certain extent, functional recovery ([Silver and Miller, 2004](#)).

Subsequently, in adolescence, CSPGs are condensed as PNNs, which are largely known to lock the synapses to prevent further modifications and close the critical period of heightened neuroplasticity as discussed in the previous section.

Intriguingly, the plasticity reinstates in the adult CNS when PNNs are disrupted. Mechanistically, disruption of PNN or its constituents triggers several short and long-term cellular and molecular changes which can promote neuroplasticity. For example, PNN depletion induces synaptic potentiation in otherwise plasticity-resistant CA2 synapses ([Carstens et al., 2016](#)). Brevican, a CSPG in the PNNs, is suggested to regulate the localization of potassium channels and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors expression on PV cells ([Favuzzi et al., 2017](#)). At the network level, PNN depletion affects gamma oscillations (30–80 Hz) ([Lensjø et al., 2017b](#)) and sharp wave ripples (SWRs) ([Sun et al., 2018](#)). Since PV neuron activity is pivotal for the generation of gamma oscillations and SWRs, it is plausible that neuroplasticity upon PNN depletion is partly effectuated by the altered activity of PV neurons. These functional changes due to PNN disruption are also accompanied by structural changes at synapses, including alterations in the numbers of synaptic contacts, spine dynamics, and expression of ion channels and receptors ([Frischknecht et al., 2009](#); [Favuzzi et al.,](#)

2017; Carceller et al., 2020). These studies suggest a variety of ways by which PNN disruption can effectuate the synaptic plasticity.

Perineuronal nets and interstitial matrix are by and large composed of the same set of ECM molecules; therefore several functions of PNNs can be considered independent of their structural integrity. However, there are several functions which require the organized PNN assembly with sulfated proteoglycans (Miyata and Kitagawa, 2016; Fawcett et al., 2019). Several signaling proteins including OTX2, Semaphorin 3a, Narp, and reelin are trapped in the PNN lattice and activate intracellular signaling cascades to facilitate the developmental maturation of PV neurons (Fawcett et al., 2019). The condensed PNN has a high density of negative charge which protects PV neurons from extracellular stressors (Suttkus et al., 2014), which is markedly evidenced in schizophrenia (Cabungcal et al., 2013, 2014) and AD wherein PV neurons are relatively spared due to their PNN coats (Morawski et al., 2010, 2012).

The sulfated proteoglycans on the PNNs constitute a high-density cloud of negative charges around the PV cells which can attract a high concentration of Na^+ , K^+ , or Ca^{++} ions. During the fast-spiking activity of PV neurons, a dynamic exchange of Na^+ and K^+ ions with the stationary negative charges of the PNNs can aid the PV neuron activity (Härtig et al., 1999; Morawski et al., 2015). Besides the

ion buffering in the ECS, PNNs can also directly influence the spiking properties of the PV neurons as shown by us ([Tewari et al., 2018](#)) and others ([Balmer, 2016](#); [Wingert and Sorg, 2021](#)). The pioneering *in vitro* ([Dityatev et al., 2007](#)) and more recent studies on hippocampal fast-spiking interneurons *in situ* brain slices ([Favuzzi et al., 2017](#); [Hayani et al., 2018](#)) report a lower firing threshold without any changes in passive neuronal properties, however, few other studies show a reduced spiking upon PNN disruption ([Balmer, 2016](#); [Tewari et al., 2018](#)). This ambiguity can be attributed to several factors including PNN disruption methods, brain regions, experimental design, resting state, and excitatory/fast-spiking type of PNN-expressing neurons, as discussed in detail by [Wingert and Sorg \(2021\)](#).

In a mouse model of human glioma-associated epilepsy, we observed that disruption of cortical PNNs by glioma-released matrix metalloproteinases (MMPs) increases the membrane capacitance of the PV neurons leading to a reduction in spike firing activity and consequently reducing the overall inhibitory drive. Experimental disruption of PNNs mimics the increased capacitance and reduced firing activity of PV neurons as shown by PV neurons with disrupted PNNs in glioma ([Tewari et al., 2018](#)), suggesting a pivotal role of PNNs in aiding the fast-spiking properties of PV neurons. PNNs seem to determine the activity of excitatory neurons equally well, as evidenced in a recent study in which microglia-mediated degradation of PNNs around excitatory projection neurons in the spinal

cord enhances their activity and induces pain-related behavior ([Tansley et al., 2022](#)). Another example is the induction of synaptic plasticity in CA2 neurons upon their PNN depletion, which are otherwise resistant to potentiation ([Carstens et al., 2016](#)).

The necessity of PNNs in CNS functioning is profoundly evidenced in CNS disorders in which PNN disruption is commonly observed; experimental PNN disruption largely phenocopies the disease characteristics. In acquired forms of epilepsies triggered by injury, stroke, and brain tumors, elevated matrix remodeling proteases disrupt the PNNs ([McRae et al., 2012](#); [Rankin-Gee et al., 2015](#); [Kim et al., 2016](#); [Dubey et al., 2017](#); [Tewari et al., 2018](#); [Patel et al., 2019](#)). PNN disruption potentially exposes the PV neurons to heightened oxidative stress ([Cabungcal et al., 2013](#)), leading to a reduction in the overall abundance of PV neurons and thereby further lowering the inhibitory drive as shown by us ([Tewari et al., 2018](#)) and others ([Enwright et al., 2016](#); [Hatcher et al., 2020](#)). Elimination of PNN and its constituents not only increases the propensity of seizure and epileptiform activity in excitatory neurons, but also causes spontaneous seizures ([Arranz et al., 2014](#); [Rempe et al., 2018](#); [Tewari et al., 2018](#); [Patel et al., 2019](#)). These studies support the idea that PNN disruption is not only able to generate neuronal hyperexcitability, but that PNN disruption due to CNS insults can

contribute to the process of epileptogenesis by PV neuron dysfunction and altered inhibition.

A similar dysfunction of PV neurons accompanied by disrupted PNNs is evidenced in animal models and human subjects of schizophrenia, bipolar disorder, and autism spectrum disorders (Pantazopoulos and Berretta, 2016; Fawcett et al., 2019), wherein preventing PNN disruption by blocking MMP activity largely ameliorates disease symptoms (Levkovitz et al., 2009; Khodaie-Ardakani et al., 2014). Several recent studies on CNS disorders including AD (Abbott and Kepler, 1990; Fawcett et al., 2019; Crapser et al., 2020b), HD (Crapser et al., 2020a, 2021), multiple sclerosis (MS) (Lau et al., 2013), and schizophrenia (Pantazopoulos et al., 2010; Mauney et al., 2013) also show altered PNN in key brain areas. Notably, glial contribution in PNN remodeling is explicitly evidenced in many of these studies as discussed in later sections.

In summary, a large number of studies suggest that the physiological functions of the PNNs and their constituents broadly encompass developmental signaling, regulation of neuroplasticity, modulation of neuronal activity, extracellular ion homeostasis, and neuroprotection. In diseased states increased proteolytic cleavage of PNNs disrupts their structural integrity and reduces the overall abundance. Depending on the brain area/s involved, loss of PNNs can contribute to disease

etiology predominantly by PV neuron dysfunction and altered E-I balance, loss of neuroprotection, altered ECS and ionic balance, and maladaptive neuroplasticity (Reichelt et al., 2019). The causal role of PNNs in E-I imbalance and ECM and ionic homeostasis in epilepsy appears to be convincing; meanwhile, the causal role of PNNs in many neuropsychiatric and neurodegenerative disorders is still in its infancy. A vast majority of the studies on the PNNs use ChABC or hyaluronidase enzymes which indiscriminately cleave the GAGs of PNNs and interstitial matrix. This lack of tools to selectively manipulate PNNs is a major limitation in the field.

Chapter4

Homeostatic regulation of extracellular matrix and perineuronal net by central nervous system glia

One of the classic housekeeping functions of glial cells is the continuous secretion of ECM molecules to maintain the architecture and extracellular milieu of the CNS. In principle, both neurons and glial cells synthesize and secrete ECM molecules; however, glial cells- especially astrocytes and microglia- are the primary regulators of ECM wear and tear in CNS pathophysiology. In this section, we discuss the role of glial cells in the homeostatic regulation of ECM and PNN and consequently the functional outcome.

Astrocytes, the most abundant CNS glia, are involved in a variety of functions including neuronal migration, secretion of growth factors and neuromodulatory molecules, synaptogenesis, synaptic pruning, and water, ion, and neurotransmitter homeostasis (Phatnani and Maniatis, 2015; Patel et al., 2019; Alcoreza et al., 2021). In the developing brain, astrocytes are the predominant source of ECM molecules including CSPGs, HA, and tenascins, which in turn serve both structural and signaling roles (Figures 2, ,3). By varying the spatiotemporal expression of CSPGs and Tn-C, astrocytes regulate the proliferation, maintenance, and

maturation of neuronal precursor stem cells and oligodendrocyte precursor cells (OPCs) as well as neuronal migration, neurite outgrowth, extension, and guidance ([Powell et al., 1997](#); [Powell and Geller, 1999](#); [Wiese et al., 2012](#); [Amin and Borrell, 2020](#); [Somaiya et al., 2022](#)). Tn-C appears to be expressed by radial glia as well as differentiated astrocytes, and additionally regulates the proliferation of astrocyte progenitor cells ([Karus et al., 2011](#)). In a quadruple knockout mouse, astrocyte-derived Tn-C, Tn-R, brevican, and neurocan have been reported to control synapse formation and stabilization ([Geissler et al., 2013](#); [Figure 3](#)).

Astrocytes are the predominant source of hyaluronan that constitutes the interstitial matrix and PNNs in the gray matter and surrounds the myelinated fibers in white matter ([Cargill et al., 2012](#); [Peters and Sherman, 2020](#)). Since hyaluronan chains of the PNN extend from the neuronal surface-bound HAS ([Fawcett et al., 2019](#)), neurons appear to be a predominant source of PNN-forming hyaluronan.

Generally, hyaluronan is secreted as a high molecular weight (HMW) polymer which interacts with HA receptors such as CD44, RHAMM, or LTR2; and triggers proliferation, differentiation, and migration of the stem cell in developing brain ([Lindwall et al., 2013](#); [Peters and Sherman, 2020](#)). In the adult brain, astrocytes produce hyaluronan as well as hyaluronan-cleaving enzyme hyaluronidase in the subventricular zone, where adult stem cells reside. Thus, by regulating the HA catabolism, astrocytes are speculated to keep stem cells in a quiescent state

([Lindwall et al., 2013](#)). In pathologies such as trauma and injury, HMW hyaluronan can be cleaved into short fragments of low molecular weight by hyaluronidases and MMPs ([Peters and Sherman, 2020](#)), which by and large have distinct and sometimes opposite biological effects. By expressing both hyaluronidase and MMPs, astrocytes play an important role in hyaluronan synthesis and catabolism ([Muir et al., 2002](#); [Al'Qteishat et al., 2006](#)). HA in turn regulates the morphology of astrocytes and proper trafficking and function of glutamate transporters by CD44-evoked Rac1 signaling ([Hayashi et al., 2019](#); [Peters and Sherman, 2020](#)).

Besides CSPGs, tenascins, and HA, which act as both structural and signaling molecules, astrocytes secrete several ECM glycoproteins known as matricellular proteins primarily for a signaling function. By adjusting the spatiotemporal expression of matricellular proteins such as hevin/SPARC ([Kucukdereli et al., 2011](#)), thrombospondin (TSP) ([Christopherson et al., 2005](#)), and Glypican 4 and 6 ([Allen et al., 2012](#)), astrocytes control excitatory synaptogenesis in the developing CNS ([Figure 3](#)). The expression of the majority of matricellular proteins decreases during the later phase of postnatal development; however reactive astrocytes upregulate their expression in several pathological conditions, potentially causing aberrant synaptogenesis and maladaptive plasticity ([Jones and Bouvier, 2014](#); [Kim et al., 2016](#)).

From a structural point of view, astrocytes, in conjunction with capillary endothelial cells and pericytes, synthesize and assemble ECM components to form the BM ([Yao et al., 2014](#); [Thomsen et al., 2017](#)). Subsequently, astrocytic perivascular endfeet- in association with the BM and pericytes- form the structural basis of the BBB, for whose maintenance astrocytic laminin is indispensable ([Thomsen et al., 2017](#)). In the parenchymal space, astrocytes secrete hyaluronan, CSPGs- including brevican, neurocan, versican, and aggrecan- and Tn-C in order to form and maintain interstitial matrix ([Wiese et al., 2012](#); [Patel et al., 2019](#)).

Although astrocytes and neurons produce aggrecan, which in turn orchestrates PNNs, the contribution of astrocytic aggrecan in PNN formation and maintenance can be questioned ([Song and Dityatev, 2017](#)) as evidenced by the absence of PNNs in neuron-specific aggrecan knockout ([Rowlands et al., 2018](#)) and formation of ECM coatings *in vitro* without astrocytes ([Miyata et al., 2005](#); [Giamanco and Matthews, 2012](#)). However, how critical astrocytes are in maintaining the PNNs *in vivo* is a frontier area of investigation. A recent study suggests that astrocytes are key regulators of PNN maturation and thereby critical period plasticity. During the critical period, immature astrocytes increase connexin 30 levels, which subsequently activates the RhoA-ROCK pathway to suppress MMP-9 expression and allows PNN condensation and PV cell maturation ([Ribot et al., 2021](#)). These

studies suggest that although astrocytes do not directly synthesize PNNs, they indirectly regulate their developmental formation.

Other CNS glial cells such as OPCs and oligodendrocytes, and microglia are also a source of ECM, but in a more restricted manner (Pu et al., 2018). For example, oligodendrocytes and OPCs deposit a peculiar ECM rich in Tn-C and Tn-R around the node of Ranvier termed the perinodal ECM, which is essential for the clustering of Na⁺ channels (Susuki et al., 2013; Fawcett et al., 2019). OPCs secrete brevican during myelination and are suggested to form axonal coats around myelinated axons (Pantazopoulos et al., 2022). Besides being a source of CSPGs, oligodendrocyte lineage cells are critically susceptible to CSPGs as evidenced by inhibition of OPCs migration and maturation into oligodendrocytes especially near the demyelinated lesion (Lau et al., 2013). Microglia can, under special circumstances, produce ECM molecules including CSPGs; however, their contribution to the formation of brain ECM and PNNs is not entirely clear (Lau et al., 2013; Pu et al., 2018). These studies suggest that glial cells are major sources of ECM molecules whereby ECM constituents are used as building blocks of brain architecture as well as signaling molecules to regulate brain development. How glial cells remodel ECM in CNS pathologies and the consequences of said remodeling are discussed in the next sections.

Extracellular matrix and perineuronal net remodeling by astrocytes in central nervous system pathologies

In nearly all CNS pathologies, astrocytes respond by undergoing morphological, molecular, and functional changes that turn them into reactive astrocytes ([Escartin et al., 2021](#)). Upregulation of glial fibrillary acidic protein (GFAP), an intermediate filament protein, is the most common molecular change and has been used widely as a marker for reactive astrocytes ([Patel et al., 2019](#); [Escartin et al., 2021](#)). In a broader sense, reactive astrocytes influence ECM and PNN homeostasis by altering the expression levels of ECM as well as the matrix remodeling proteases including MMPs. As a consequence, in several CNS pathologies such as acquired epilepsies, TBI, MS, and glioma, the interstitial matrix is upregulated; however, the PNNs are disrupted or lost ([Lau et al., 2013](#); [George and Geller, 2018](#); [Rempe et al., 2018](#)). This contrasting fate can be attributed to the glial upregulation of ECM molecules at the injury and increased expression of matrix-degrading proteases in the surrounded areas. Interestingly, CNS disorders without a focal injury such as schizophrenia also show glial ECM abnormalities in conjunction with PNN disruption in several key brain areas suggesting a potential role of glia in PNN

reduction ([Pantazopoulos et al., 2010, 2015](#); [Mauney et al., 2013](#)). Considering its widespread prevalence, ECM remodeling appears to be a generic response of reactive astrocytes to a majority of brain disorders ([Figure 3](#)).

The deleterious consequences of ECM remodeling by glial cells are remarkable in CNS pathologies with a focal lesion such as ischemia, glioma, MS lesion, and brain and spinal cord injury. At the focal lesion or injury site, astrocytes turn reactive ([Escartin et al., 2021](#)) and form a barrier or glial scar, aided by infiltration of microglia, macrophages, meningeal cells, and fibroblast ([Rhodes et al., 2003](#)). Scar-forming reactive astrocytes accumulate a variety of ECM molecules, including CSPGs, HA, tenascins, fibronectins, and laminins, and isolate the insult from surrounding areas to limit the spread of inflammation and tissue damage ([Kim et al., 2016](#)). In the case of traumatic injuries, especially in the spinal cord, reactive astrocytes deposit various CSPGs in the glial scar which inhibits neuronal recovery and axonal regrowth ([Silver and Miller, 2004](#); [Lau et al., 2013](#)). Similarly, in cortical injury, reactive astrocytes highly upregulate CSPGs and inhibit cortical axonal regeneration ([McKeon et al., 1999](#); [Busch and Silver, 2007](#); [Kim et al., 2016](#)). ChABC application to dissolve CSPGs enhances axonal growth and functional recovery to great extent ([Bradbury et al., 2002](#); [Huang et al., 2006](#); [Lee et al., 2010](#)). Interestingly, astrocyte specific ChABC expression in transgenic mice reduced CSPG expression after spinal cord injury and enhanced

axonal growth and recovery ([Cafferty et al., 2007](#)). These studies provide compelling evidence of inhibitory functions of glial-derived CSPGs and can be harnessed to generate glia-centric therapeutic tools.

Reactive astrocytes also release non-sulfated proteoglycan HA in the glial scar areas in the brain or spinal cord lesions, evoking beneficial and deleterious effects ([Sherman et al., 2015](#)). The HMW HA accumulation after spinal cord injury suppresses the activation of astrocytes as well as glial scar formation. On the other hand, low MW hyaluronan accumulation [perhaps as a cleavage product of HMW HA due to increased hyaluronidase activity ([Al'Qteishat et al., 2006](#))] promotes astrocytic activation and proliferation ([Struve et al., 2005](#)). Following an ischemic stroke, scar-forming astrocytes upregulate the hyaluronan as well as HA receptor RHAMM, and are speculated to evoke the migration of adult stem cells from the subventricular niche to the ischemic site for repair ([Lindwall et al., 2013](#)). Glial upregulation of HA is also evidenced in aging; however, the downstream effects require thorough investigation ([Peters and Sherman, 2020](#)).

Similarly to CNS trauma and injury, in MS several ECM components such as hyaluronan, CSPGs (aggrecan, versican, and neurocan), and glycoprotein (fibronectin and vitronectin) are deposited around the lesion, primarily by reactive astrocytes forming the glial scar ([Lau et al., 2013](#)). Condensed CSPGs and HA at

the lesion prevent OPC growth, differentiation, and migration to the lesion site, thereby making remyelination nearly impossible. Despite providing beneficial effects to the axon growth in spinal cord injury, HA and CSPG disruption strategies using exogenous MMPs, ChABC or hyaluronidase have not proven promising, largely due to indiscriminate disruption of both permissive and non-permissive ECM components and generation of non-permissive cleavage product (Lau et al., 2013). Since reactive astrocytes are the major CSPG and HA-producing cells at the lesion, strategies to manipulate the cellular machinery of CSPG and HA biosynthesis and release can be speculatively proposed as an alternative approach.

Since PNN disruption is commonly observed in several CNS disorders, it is a legitimate question whether astrocytes play any role in PNN disruption in such pathologies. The answer lies partly in the fact that astrocytes are a major source of PNN-disrupting MMPs in both homeostatic and diseased states. After brain injury and trauma, PNN disruption is accompanied by upregulation of MMP-2 and MMP-3 levels by reactive astrocytes (Muir et al., 2002; Falo et al., 2006; Lorenzo Bozzelli et al., 2018). MMP-9 appears to be the major effector of PNN disruption triggered by activation of TGF β signaling in reactive astrocytes after albumin extravasation due to BBB disruption in various neurological insults (Kim et al., 2017). Notably, microglia, infiltrating immune cells, and neurons are also sources of MMPs after CNS insults; therefore astrocytes may not be solely responsible for

PNN disruption ([Rosenberg, 2002](#)). This fact is also supported by our study on glioma-associated epilepsy, in which PNN disruption did not spatially correlate with reactive astrogliosis and astrocytes did not show MMP activity ([Tewari et al., 2018](#)).

Extracellular matrix and perineuronal net remodeling by microglia in central nervous system pathologies

Microglia are the resident immune cells in the brain and are known for their classic phagocytic function of eliminating entire cells or subcellular structures- mainly synapses- in healthy and diseased states ([Wolf et al., 2017](#)). The developing brain forms an excessive number of synapses, which are subsequently eliminated by a process known as synaptic pruning, in which microglia play a pivotal role. In a healthy adult brain, microglia also actively prune synapses in an effort to aid neuronal plasticity ([Wolf et al., 2017](#)). Besides these well-known functions, recent studies suggest a conversion of normal astrocytes to a detrimental neurotoxic phenotype under the influence of microglia-released cytokines IL-1 α , TNF- α , and complement protein C1q in pathological conditions ([Liddelow et al., 2017](#)).

Pioneering studies in the recent past implicate microglia equally well in the regulation of homeostatic functions such as ECM and PNN remodeling, eventually governing synaptic plasticity. The classic example is a reversible increase in the abundance and condensation of cortical PNNs ([Crapser et al., 2020a](#); [Liu et al., 2021](#)) and increased brevican accumulation around hippocampal synapses upon elimination of microglia in normal CNS ([Strackeljan et al., 2021](#)). PNN and ECM disruption is evidenced in CNS pathologies such as AD, wherein microglia appear to effectuate the clearance of the ECM and PNN components either directly, by stripping PNNs from the neuronal surface; or indirectly, by clearing the PNN debris after proteolytic cleavage by MMPs ([Fawcett et al., 2019](#); [Crapser et al., 2020b,2021](#)). Interestingly, eliminating microglia effectively restores the ECM components as well as PNN ([Crapser et al., 2020a,b](#)) and can be explored further from a therapeutic angle.

A similar role of microglia has recently been reported in the spinal cord dorsal horn wherein peripheral nerve injury promotes microglial degradation and endocytosis of PNNs around projection neurons. PNN depletion activates the projection neurons to induce pain-related behavior ([Tansley et al., 2022](#)). Despite such explicit presentations of microglial involvement, what triggers microglia to start stripping PNNs and ECM components with or without proteolytic cleavage is still an open question. At a minimum, it can be speculated that homeostatic PNN

regulation can also be regulated by neuronally released cytokine interleukin-33 (IL-33) which guides microglia to clear ECM to facilitate homeostatic synaptic plasticity; a loss of IL-33 signal leads to ECM accumulation around synapses and dendrites ([Nguyen et al., 2020](#)). Similarly, in the developing brain, astrocytes are the main source of IL-33, which in turn instructs microglia to engulf redundant synapses ([Vainchtein et al., 2018](#)). Another study revealed the role of microglial protease cathepsin-S in maintaining a diurnal rhythm of the PNN labeling by demonstrating modification in PNNs in a circadian manner that coincides with the rhythmic expression of protease cathepsin-S ([Pantazopoulos et al., 2020](#)); however, cyclic rhythm of PNNs is still debated ([Barahona et al., 2022](#)). Collectively these and several other recent studies ([Venturino et al., 2021](#)) suggest microglia as an integral element of the ECM and PNN remodeling in homeostasis and diseased states; however, the mechanistic insight and therapeutic potential warrant further investigation.

Metalloproteinases: Main effectors of extracellular matrix and perineuronal net remodeling

Despite a wide range of upstream cellular and molecular events, the mechanisms of ECM and PNN remodeling largely converge onto the metalloproteinases, which

serve as an immediate effector to decisively control the ECM and PNN remodeling. Metalloproteinases are zinc-containing endopeptidases, normally expressed by neurons, astrocytes, microglia, and endothelial cells. However, under pathological conditions such as ischemic stroke and brain tumors, non-resident immune cells and tumor cells can also produce metalloproteinases ([Tewari et al., 2018](#); [Patel et al., 2019](#); [Chaunsali et al., 2021](#)). Proteolytic remodeling of ECM by metalloproteinase is pivotal in regulating multiple physiological and pathophysiological processes during CNS development and survival, angiogenesis, neurogenesis, axonal growth and regeneration, CNS injury, tumor metastasis, and neuroinflammation ([Gottschall and Howell, 2015](#); [Rempe et al., 2016](#)).

Metalloproteinases consist of two families: MMP and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs). In principle, out of 24 MMPs, MMP-1–3, 7–17, 24, and 28 are expressed in the brain under specific pathophysiological circumstances; however, a vast majority of them remain largely undetectable in a normal physiological state ([Rempe et al., 2016](#); [Beroun et al., 2019](#)). Based on substrate specificity, major MMPs can be categorized into collagenase (MMP-1, 8, 13); gelatinase (MMP-2, 9) which can degrade collagen IV, fibronectin, laminin, aggrecan, gelatin, elastin, and non-matrix substrates; and stromelysins (MMP-3, 10, 11) which can cleave collagens I, III, IV, V, IX, and X ([Nakamura et al., 2009](#)), fibronectin, denatured collagens, laminin, and cartilage

proteoglycans ([Chang et al., 2016](#)). ADAMTSs are equally diverse with 19 family members, of which ADAMTSs 1, 4, 5, 9, and 15 are expressed in specific brain regions under different circumstances ([Gottschall and Howell, 2015](#)). ADAMTSs 1, 4, and 5 are known as proteoglycanases or aggrecanases as they can cleave major brain proteoglycans aggrecan, brevican and versican ([Kelwick et al., 2015](#)). By and large, ADAMTSs complement the homeostatic and pathological functions of MMPs in developmental ECM remodeling, tissue repair after injury, inflammation, and cancers ([Song and Dityatev, 2017](#); [Testa et al., 2019](#)).

Matrix metalloproteinases and ADAMTSs can collectively cleave all elements of the ECM and PNNs as well as several growth factors and membrane proteins (See review [Yong et al., 2001](#); [Lu et al., 2011](#); [Gottschall and Howell, 2015](#)); thus, their upstream and downstream regulations are critically important. Indeed, the activity of metalloproteinase is tightly regulated at multiple levels, which makes them ideal candidates for spatiotemporally controlling the ECM and PNN remodeling. At the transcriptional level, most of the MMPs are expressed non-constitutively and need some form of extrinsic signals to trigger their transcription, such as inflammatory cytokines, growth factors, chemokines, and cell-cell or cell-matrix interactions. Several MMPs are expressed as inactive zymogens and need an activation process; this is predominantly cleavage of a propeptide region by intra- and extracellular proteases, including other MMPs. These post-translation modifications bring

another level of MMP activity regulation. Even after MMPs are synthesized, secreted, and activated, tissue inhibitors of metalloproteinases (TIMP1-4) can inactivate them by reversibly binding to their catalytic site and thereby terminating the proteolytic activity (Yong et al., 2001). Among other extrinsic regulators, activation of neurotransmitter receptors for glutamate (Dziembowska et al., 2012), dopamine (Mitlöhner et al., 2020), and serotonin (Bijata et al., 2017) are shown to directly regulate MMP-9 and ADAMTS-4 and -5 activity and subsequently trigger neuroplasticity.

From the brain ECM perspective, MMP-2 and MMP-9, and to a lesser extent MMP-3, are the most studied metalloproteinases, perhaps due to their reliable detection and measurement methods and also their involvement in multiple processes (Dzwonek et al., 2004). MMP-2, or gelatinase A, is expressed by both neurons and glial cells; however, astrocytes appear to be the major source (Dzwonek et al., 2004; Brkic et al., 2015). Functionally, mostly MMP-2, along with MMP-9, is involved in several processes including neurogenesis, migration, dendritic and axonal outgrowth, and guidance in the developing brain as well as migration, regeneration, adult neurogenesis, and angiogenesis in the adult brain (see review Verslegers et al., 2013). In several CNS pathologies in which PNN disruption and ECM remodeling is commonly observed- including ischemia, traumatic brain injury, and seizures- MMP-2 levels, especially in glial cells, are

also upregulated (Dzwonek et al., 2004; Kim and Hwang, 2011; Verslegers et al., 2013; Quattromani et al., 2018). Despite such explicit correlation, the decisive contribution of astrocytic MMP-2 in PNN disruption is hard to discern due to a concomitant release of MMP-9 from neurons which can also degrade PNNs (Kim and Hwang, 2011).

MMP-9 is another gelatinase expressed by neurons, astrocytes, and oligodendrocytes (Kamat et al., 2014; Song and Dityatev, 2017) and is the most widely implicated metalloproteinase in CNS homeostasis and pathologies. MMP-9 appears to be the key player in ECM and PNN remodeling-induced neuroplasticity in physiological and pathological conditions. For example, MMP-9 and MMP-2 loosen PNN after enriched environment rearing (Foscarin et al., 2011) or light exposure after monocular deprivation (Wen et al., 2018b) to facilitate neuroplasticity. MMP-9 plays a pivotal role in hippocampal-dependent plasticity, possibly by ECM degradation and inducing surface recruitment of NMDAR between non-synaptic and synaptic compartments, activation of L-VDCCs, and dendritic spine modifications (Kochlamazashvili et al., 2010; Verslegers et al., 2013). A recently reported fundamental role of astrocytes in closing the critical period of visual plasticity is effectuated by MMP-9. Astrocytic connexin 30 signaling suppresses MMP-9 expression to allow maturation of PNNs, thereby closing the critical period of visual plasticity (Ribot et al., 2021). On the other

hand, reactive astrocytes in CNS pathologies upregulate MMP-9 *via* inflammatory signaling ([Kim et al., 2016](#)). MMP-9 upregulation correlates remarkably with disruption of the PNN in an overwhelming number of studies on epilepsy ([McRae and Porter, 2012](#); [Kim et al., 2016, 2017](#); [Tewari et al., 2018](#); [Chaunsali et al., 2021](#)), TBI and stroke ([Kim et al., 2017](#)), glioblastoma ([Tewari et al., 2018](#); [Hatcher et al., 2020](#)), neurodegenerative ([Kamat et al., 2014](#); [Brkic et al., 2015](#)) and neuropsychiatric diseases, and blocking MMP-9 activity generally prevents PNN disruption and improves the disease pathologies ([Testa et al., 2019](#)). More recently, inhibition of MMP-2/9 by IPR-179 showed promising antiseizure and antiepileptogenic effects as well as improved cognitive deficits induced by seizures ([Broekaart et al., 2021](#)). Similarly, in genetic disorders including Fragile X Syndrome, elevated MMP-9 correlates with PNN disruption, and genetic reduction of MMP-9 promotes PNN formation ([Wen et al., 2018a](#)). Overall, MMP-2 and MMP-9 are primary effectors of neuronal and glial cell mediated ECM and PNN remodeling in health and diseases.

MMP-3 or stromelysin-1 is an emerging ECM regulator due to its broad substrate specificity, particularly in its ability to activate pro-MMPs including MMP-2 and MMP-9 ([Kim and Hwang, 2011](#)). Perhaps due to this upstream control ability, MMP-3 level in healthy neurons is very low to undetectable. In pathological conditions, neurons, oligodendrocytes, astrocytes, and microglia release MMP-3,

which is implicated in neuroinflammation and apoptosis associated with several neurodegenerative disorders (Rempe et al., 2016). Compared to MMP-9, the roles of MMP-3 in context to PNN remodeling and neuroplasticity in adult brains are not explicitly studied. However, MMP-3 has largely been associated with detrimental processes including inflammation, apoptosis, BBB breakdown, neurodegeneration, and demyelination (Kim and Hwang, 2011; Van Hove et al., 2012).

Besides metalloproteinases, CSPGs and PNNs can also be cleaved by tissue-type plasminogen activator (tPA) which is a serine protease and classically known to dissolve blood clots. tPA can degrade PNN directly as its substrate or *via* activating MMPs and upregulated tPA levels may be associated with disrupted PNNs in ischemia and epilepsy (McRae and Porter, 2012; Quattromani et al., 2018). However, whether the homeostatic tPA expression can remodel PNNs is not known. Endogenous hyaluronidase is another enzyme that can degrade PNNs indirectly by cleaving the hyaluronan backbone of the PNNs. Although hyaluronidase levels are elevated after stroke and brain injury, not much is known about their role in the homeostatic and pathological remodeling of ECM and PNNs (Sherman et al., 2015; Reed et al., 2019).

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Chapter5

Epilepsy is a type of neurological disorder characterized by symptoms of periodic, unpredictable, and recurrent seizures ([Beghi et al., 2019](#)). According to the International League Against Epilepsy (ILAE) classification in 2017, epilepsies are classified as focal, generalized, combined generalized and focal epilepsy or

unknown types ([Scheffer et al., 2017](#)). Although the cause of epilepsy is still unknown in most cases, seizures can be caused by any damage associated with metabolic disorders, infectious diseases, genetic mutation, or immune disorders that disrupt brain function ([Devinsky et al., 2018](#)).

Currently available antiepileptic drugs (AEDs) are only partially efficient for epilepsy. Although AEDs control nearly 70% of patients with epilepsy, 5%–10% of patients still require additional medications and more than 20% of patients continue to have seizures after treatment. They can only treat symptoms of epilepsy without making any significant progress in neither reversing its underlying mechanisms nor offering neuroprotection caused by the pathogenesis of epilepsy, resulting in about 15%–40% of all patients with epilepsy, unfortunately, continuing to have seizures that impair their daily living ([Sankaraneni and Lachhwani, 2015](#)). Moreover, most AEDs have shown cognitive, psychiatric, and behavioral side effects on patients ([Chen et al., 2017](#)). For example, cognitive abnormality, especially impairment in learning and memory, is one of the serious comorbidities caused by epileptic seizures and/or AEDs that has a significant negative impact on patients' quality of life ([Holmes, 2015](#)). The neuronal damage and death caused by seizure-associated excitotoxicity are most likely responsible for both recurrent epilepsy itself and epilepsy-induced cognitive impairment. Therefore, dampening excitotoxicity, which is resulted from an imbalance between

the excitatory and inhibitory neurotransmitters, was the main target of currently available AEDs (Sankaraneni and Lachhwani, 2015).

Since the current anti-epileptic treatment is not ideal, the development of more effective and clinically relevant novel therapeutic approaches for epilepsy is an urgent need and hope. In recent years, more and more evidence has shown that oxidative stress and neuroinflammation play important roles in the pathophysiology of acquired epilepsy (Vezzani, Balosso, and Ravizza, 2019). In this respect, oxidative stress and neuroinflammation pathways could be desirable therapeutic targets in epilepsy. Previous studies have demonstrated that the use of plant extracts and plant-based antioxidant and anti-inflammatory compounds such as flavonoids could reduce or eliminate neuroinflammation and oxidative damage (Kwon et al., 2019). One such well-studied herbal plant family is the genus *Artemisia*. Many phytochemical and pharmacological studies have reported that the genus *Artemisia* contains a huge amount of interesting polyphenolic constituents and showed promising anticonvulsant properties in experimental studies using various animal models of epilepsy through various mechanistic pathways. However, the detailed literature on the antiepileptic properties of the genus *Artemisia* and its mechanism of action is segregated. In this review, we tried to gather the detailed neuroprotective and antiepileptic properties of the genus *Artemisia* and its possible underlying mechanisms.

In this mini-review, we have explored the therapeutic potential of *Artemisia* for the treatment of epilepsy. Data were collected by searching relevant original articles using the scientific databases Pubmed and Google scholars with the keywords “artemisia,” “neuroprotection,” “anticonvulsants” or “anti-epileptic drugs.” As a result, 63 articles were retrieved, of which 18 original research articles were reviewed based on the pharmacological use of *Artemisia* in epilepsy. This review discusses current research on certain species of the genus *Artemisia* with anti-epileptic and neuroprotective activities. In addition, the anti-epileptic mechanisms of their bioactive components and the experimental models used in studies are also introduced and discussed.

2 The redox-associated neural apoptosis and its role in epilepsy

Oxidative Stress (OS) might play a role in epilepsy and associated cognitive disorders (Geronzi, Lotti and Grosso, 2018). A growing number of studies in cellular and animal models have shown that OS is involved in the development of recurrent seizures, status epilepticus (SE), and learning abnormalities by causing neural damage and death (Vilar Da Fonsêca et al., 2019; Eastman, D’Ambrosio and Ganesh, 2020; Lin et al., 2020; Golub and Reddy, 2022). However, the exact

mechanisms by which how OS can provoke a normal brain to develop spontaneous seizures and cognitive comorbidities have not been completely elucidated. Some experimental evidence revealed an association between neuronal apoptosis and epileptogenesis whilst it is unclear whether apoptosis is a cause or a consequence of epilepsy.

Apoptosis is primarily induced by two main pathways—the extrinsic and intrinsic pathways. In the extrinsic pathway, apoptosis is initiated by the activation of death receptors, whereas, in the intrinsic pathway, it is induced by the activation of a series of apoptotic protein that changes mitochondrial permeability, leading to the release of cytochrome c into the cytosol (Bedoui, Herold and Strasser, 2020). The Bcl-2 family is a well-characterized protein family involved in the modulation of apoptotic cell death, consisting of anti-apoptotic Bcl-2 protein and pro-apoptotic Bax and Bak, which are key modulators of the intrinsic (mitochondrial) pathway of apoptosis. Under normal conditions, Bcl-2, which localizes to the outer mitochondrial membrane, promotes cell survival by inhibiting Bax/Bak oligomerization, which would otherwise facilitate the release of cytochrome c from mitochondria (Ge and Wang, 2020). In response to cellular stress, Bax/Bak oligomers create pores in the mitochondrial outer membrane, leading to depolarization of the mitochondrial membrane potential ($\Delta\Psi_m$) and release of cytochrome c into the cytoplasm (Ly, Grubb and Lawen, 2003; Westphal et al.,

2011). Cytosolic cytochrome c then triggers apoptosis by binding to Apaf-1 (apoptotic protease activating factor 1) and procaspase-9 that further activates downstream caspases (Westphal et al., 2011).

Neuronal mitochondria—the primary sites of reactive oxygen species (ROS) generation in the brain—are particularly sensitive and vulnerable to oxidant damage by ROS during epileptic seizures. Mitochondrial oxidative stress (MOS) caused by ROS overproduction is the main culprit that triggers diverse cell death pathways (Zhao et al., 2020). Several factors are likely to be implicated in the modulation of MOS, neuronal death, and epilepsy (Figure 1).

2.1 Apoptosis

The activation of both extrinsic and intrinsic apoptotic signaling pathways has been reported to exacerbate seizure-induced brain damage and leads to SE. An excessive and/or consistent neuronal excitation associated with seizures causes increased production of ROS, the liberation of cytochrome c from mitochondria, leading to activation of downstream caspases (e.g., caspase-3) and eventually neuronal apoptosis (Engel and Henshall, 2009).

2.2 mtDNA damage

Increased production of ROS by epileptic seizures also causes mtDNA damage that can disrupt normal functions of mitochondrial ETC thus reduce ATP supply to the neural tissue ([Jarrett et al., 2008](#)).

2.3 Mitochondrial fission

Seizures-induced oxidative stress accelerates mitochondrial fission, increases expressions of cytochrome c and caspase-3 as well as reduces neuronal viability ([Qiu et al., 2013](#)).

2.4 Mitochondrial Ca^{2+} accumulation

Seizure-induced stress on neurons enhances accumulation of mitochondrial Ca^{2+} , which can not only further stimulate overproduction of ROS, but also facilitate the release of cytochrome c from the mitochondria to cytosol, triggering caspase-3-dependent neural apoptosis ([Fricker et al., 2018](#); [Ge and Wang, 2020](#)).

2.5 Neuroinflammation

There is also evidence supporting the contribution of MOS to neuroinflammation in epilepsy. In various animal models of epileptogenesis, a typical

neuroinflammatory response characterized by robust astrogliosis, microglial activation, and the production of cytokines and chemokines has been observed and associated with neuroinflammation ([Pauletti et al., 2019](#); [Vezzani, Balosso and Ravizza, 2019](#); [Terrone et al., 2020](#)).

These data suggest that MOS is likely an important pathogenic factor of epilepsy and targeting MOS and the intrinsic mitochondrial pathway might be a novel strategy to suppress neuronal apoptosis and alleviate epileptogenesis. In line with this assumption, Coenzyme Q10 (CoQ10)—an antioxidant produced naturally by our body—has evident neuroprotection against pentylenetetrazol (PTZ)-induced kindling *via* suppressions of oxidative damage and neuroinflammation ([Bhardwaj and Kumar, 2016](#)). Moreover, Co Q10 levels in epilepsy patients were significantly lower in epilepsy patients compared to healthy controls ([Simani et al., 2020](#)). To some extent, these data indirectly reflect the relationship between mitochondrial oxidative damage and neuroinflammation.

2.6 Excitotoxicity

Furthermore, oxidative stress and mitochondrial dysfunction contribute to glutamate-induced excitotoxicity and later neuronal apoptosis. The excitotoxicity—induced damage and death of hippocampal neurons are quite recurrent in cases of epilepsy, contributing to cognitive dysfunction ([Sabogal-Guáqueta et al., 2019](#)).

Taken together, these studies suggest that substances with antioxidant, anti-inflammatory, and anti-excitotoxic activities may promote neuroprotection and prevent epilepsy by reducing brain oxidative stress, neuroinflammation, neuronal apoptosis, and improving cognitive dysfunction.

3 The genus artemisia and its antiepileptic activity

The genus *Artemisia*, one of the most distributed genera in the Asteraceae family, is mostly distributed in Europe, Asia, and North America ([Abad et al., 2012](#)).

Many of them have been used since ancient times as folklore remedies for various diseases, such as malaria, fever, cough, and gut parasitic diseases ([Rasooli et al., 2003](#); [Bora and Sharma, 2011b](#)). Pharmacological studies demonstrated that *Artemisia* species possess a wide range of antioxidant, anti-inflammatory, antimalarial, antimicrobial, antiviral, antitumor, antipyretic, antihemorrhagic, anticoagulant, antianginal, antihepatitic, antiulcerogenic, and antispasmodic activities ([Abad et al., 2012](#)).

Despite their wide diversity, distribution, and application, most *Artemisia* species investigated to date have similar chemical compositions. The principal bioactive constituents found in this genus include isoprenoids, flavonoids, phenolic acids, coumarins, glycosides, sterols, polyacetylenes, and caffeoylquinic acids ([Tan, Zheng and Tang, 1998](#); [Bora and Sharma, 2011b](#)), and the presence of these

bioactive compounds in varying proportions in different plants might be attributed to *Artemisia*'s diverse pharmacological activities. For example, flavonoids, which are rich in *Artemisia annua* (Baraldi et al., 2008), possess numerous antioxidant, anti-inflammatory, and anti-apoptotic activities.

In recent years, more and more evidence has shown that oxidative stress and neuroinflammation play important roles in the pathophysiology of acquired epilepsy. A line of experimental studies has demonstrated that oxidative stress and neuroinflammation are implicated in the development of recurrent seizures and SE (Rowley et al., 2015; McElroy et al., 2017; Wang et al., 2017; Vezzani, Balosso and Ravizza, 2019) as well as cognitive abnormalities caused by recurrent seizures by inducing oxidative damage to neural tissue (Méndez-Armenta et al., 2014; Lee et al., 2017; Deng et al., 2019). Therefore, the focus of epilepsy research and treatment strategy has now shifted to seeking alternative approaches, such as herbal plants, with better efficacy and minimal side effects (Gao et al., 2014; Kwon et al., 2019; Rehman et al., 2019). It is postulated that the use of plant-based antioxidants (e.g., flavonoids) to reduce or eliminate neuroinflammation and oxidative damage might be a desirable strategy in the treatment of epilepsy and epilepsy-/AEDs-induced cognitive impairment.

Several *Artemisia* species have been used in folklore medicine to treat epileptic seizures, and the anticonvulsant efficacy of these plants was confirmed by *in vivo* animal experiments using acute and chronic epilepsy (De Lima, Morato and Takahashi, 1993; Sayyah, Nadjafnia and Kamalinejad, 2004; Mio et al., 2010; Khan et al., 2016; Kediso et al., 2021). Studies indicated that essential oil, crude extracts, and different fractionations of *Artemisia* species can prevent seizures. De Lima et al. (1993) reported earlier that the hydroalcoholic extract (HE) of *Artemisia verlotorum* prevented non-invasive electroshock (75 mA, 60 Hz) and the seizure-induced by pentylenetetrazole. In addition, HE also increased the latencies of seizures in both pilocarpine and 3-mercaptopropionic acid pilocarpine-induced mice (De Lima et al., 1993). These results suggest that extracts of *A. verlotorum* are able to protect against experimental convulsions elicited by various agents. *Artemisia dracunculus* L. is used as an antiepileptic remedy in Iranian folklore medicine. Essential oil of *A. dracunculus* L. demonstrated the anti-seizure activity in mouse models of acute epilepsy (Sayyah, Nadjafnia and Kamalinejad, 2004). The extracted *A. dracunculus* L. essential oil showed a dose and time-dependent anticonvulsant activity in both maximal electroshock (MES; ED₅₀ 0.84 ml/kg) and pentylenetetrazole (PTZ; ED₅₀ 0.26 ml/kg) mice models of seizure, respectively. After gas chromatography (GC)/mass spectrometry (MS) analysis of the essential oil, authors postulate that monoterpenoids in the essential

oil may be responsible for the anticonvulsant effects (Sayyah, Nadjafnia and Kamalinejad, 2004). The potential anti-epileptic effect of *Artemisia copa Phil.* was explored by Mio et al. (2010) as a part of their psychopharmacological studies. They evaluated the anticonvulsant activity of plant water extracts on a PTZ-induced mouse model and found that *Artemisia copa Phil.* extracts (150 mg/kg) significantly increased the latency time and decreased the duration of seizures and mortality in mice. Khan et al. (2016) studied the anticonvulsive effect of *Artemisia Indica* fractions and the involvement of GABA-A receptors using electrophysiological methods. The results showed that the isolated carnosol, ursolic acid, and oleanolic acid (10–100 mg/kg) had significant anticonvulsant activity in a PTZ-induced epilepsy mouse model by positively regulating the $\alpha 1\beta 2\gamma 2L$ GABA-A receptor activity. Kediso et al. (2021) evaluated the effect of an aqueous ethanolic extract of *Artemisia afra* on pentylenetetrazole-induced seizures in mice. They noted that extracts of *Artemisia afra* showed a delay in seizure onset and a reduction in the duration of convulsions in a dose-dependent manner.

To sum up, certain *Artemisia* species have been found to be effective anticonvulsants. However, the exact pharmacologically active compounds of the genus *Artemisia* and the underlying cellular and molecular mechanisms through which *Artemisia* exerts antiepileptic effects are not fully understood. There is increasing evidence suggesting that some *Artemisia* species might have therapeutic

potential for epilepsy due to their antioxidant, anti-inflammatory, and anti-apoptotic properties as well as their regulation of neurotransmission (Hamsalakshmi et al., 2022).

Chapter6

4 Antiepileptic mechanisms of the genus artemisia

4.1 Antioxidant activity of the genus artemisia

Oxidative stress generated by ROS may play a role in the occurrence and development of epilepsy, and changes in mitochondria-related oxidative stress status can lead to neuronal apoptosis. Various plants from the *Artemisia* species demonstrate protective activities against oxidative damage to neural tissue *in*

vitro and *in vivo*. Treatment with *Artemisia* extracts is able to protect neurons or recover the oxidative stress-induced damage by preventing the formation of free radicals, enhancing the activity of the endogenous antioxidant system, and regulating apoptosis signaling pathways.

One initial study with *Artemisia* extracts indicated that the methanol extract of *Artemisia absinthium* has free radical scavenging activity *in vitro* and antioxidant capacity in the animal brain (*in vivo*) by decreasing thiobarbituric acid reactive substances (TBARS) and restoring levels of superoxide dismutase (SOD) and glutathione (GSH) (Bora and Sharma, 2011a). Another recent study using rats showed that the levels of the pro-oxidants MDA (an indicator of lipid peroxidation) and nitric oxide (NO) were significantly reduced, while the levels of antioxidant enzymes GSH and its gene expression in cortical tissue were significantly increased in streptozotocin (STZ)-induced diabetic rats exposed to ethanol extract of *Aretemisia judaica* (Albasher et al., 2020). More recently, Rashidi et al. (2021) demonstrated that ethanolic extract of *Aretemisia absinthium* has antioxidant effect on 6-hydroxydopamine (6-OHDA)-induced oxidative stress in SH-SY5Y cells. The extract of the plant at concentrations ranging from 6.25 to 25 µg/ml has been found to significantly decrease intracellular ROS formation induced by 6-OHDA. The plant extract also increases the GSH level and SOD activity. Similarly, various fractions of two *Artemisia*

species—*Artemisia turanica* and *Artemisia turcomanica*—were found to effectively suppress H₂O₂-induced oxidative stress and apoptosis of PC12 cells as well as restored the H₂O₂-induced GSH depletion (Hosseinzadeh et al., 2018).

Besides the crude Artemisia extracts, the essential oil from *Artemisia campestris* has been shown to be a potent antioxidant (Saoudi et al., 2017).

Pretreatment of animals with the essential oil significantly reduces deltamethrin-induced oxidative damage in rat brain tissue by alleviating lipid peroxidation, oxidative stress, and degeneration of brain tissue (Saoudi et al., 2017).

The antioxidant activity of Artemisia was also investigated using secondary metabolites of the genus. Caffeoylquinic acids and their derivatives isolated from *Artemisia princeps* Pam. showed a potent antioxidant effect on β -amyloid-induced oxidative stress in PC12 cells in a dose-dependent manner. Under oxidative stress conditions, PC12 cells were treated with whole extract and 3,5-dicaffeoylquinic acid (3,5-diCQA) increased the cell viability by approximately 1.6 and 2.4 times, respectively, compared to the control without treatments (Lee et al., 2011). In addition, 3,5-diCQA isolated from *Artemisia argyi* H. also demonstrated potent antioxidant property (Kang et al., 2016). In this study, 3,5-diCQA could restore cognitive dysfunction and the antioxidant capacity in trimethyltin (TMT)-treated mice by reducing the amount of pro-oxidant malondialdehyde (MDA) and augmenting the levels of oxidized GSH in the brain tissue of ICR mice (Kang et

al., 2016). Moreover, DSF-52, a sesquiterpene dimer isolated from *Artemisia argyi*, could suppress NADPH oxidase blocking ROS production in lipopolysaccharide (LPS)-induced BV-2 cells (Zeng et al., 2014).

Finally, *Artemisia amygdalina* showed antioxidant action on differentiated N2a and SH-SY5Y cells (Sajjad et al., 2019). The oxidative stress and cell death induced by H₂O₂ in these cell lines were attenuated by different extracts of *Artemisia amygdalina* by upregulation of the Nrf2 signaling pathway, which is known to be an emerging modulatory pathway of cells resistant to oxidants (Saha et al., 2022).

From the aforementioned data, it was suggested that the antioxidant properties and underlying mechanisms of the genus *Artemisia* might be attributed to the bioactive secondary metabolites of the plants. To date, most studies associated with the antioxidant activity of *Artemisia* extracts have focused on Artemisinin—the principal bioactive isoprenoids isolated from *Artemisia annua*. A pioneering study conducted by Zheng et al. (2016) demonstrated that Artemisinin has a neuroprotective effect on sodium nitroprusside-induced oxidative damage to primary cortical neurons and PC12 cells *in vitro*. They found that pretreatment of PC12 cells with Artemisinin could protect cell viability by reducing oxidation and preventing the decline of mitochondrial membrane potential. In addition, they also demonstrated by Western blotting analysis that the neuroprotective effect of

Artemisinin is associated with the activation of the extracellular regulated protein kinases (ERK) pathway, which is responsible for intracellular signaling. The involvement of the ERK/CREB signaling pathway was also supported by other studies ([Chong and Zheng, 2016](#)), which demonstrated that H₂O₂-induced oxidative damage can be suppressed by Artemisinin in retinal pigment epithelial cells D407 through restoring cell morphology, mitochondrial membrane potential, and slowing intracellular ROS generation. In line with those findings, [Yan et al. \(2017\)](#) demonstrated that Artemisinin has a protective function against H₂O₂-induced oxidative stress in retinal neuronal cells RGC-5. In this study, a decreased ROS accumulation and cell apoptosis, as well as an increased mitochondrial membrane potential has been observed following the treatment of cells with Artemisinin. The Western blotting analysis indicated that H₂O₂-induced oxidative stress in RGC-5 cells upregulated the phosphorylation of P38 and ERK1/2 kinase pathways, which could be reversed by Artemisinin. Interestingly, they also observed that intravenous administration of artemisinin in a concentration-dependent manner protected the retinal function from light-exposed damage by measuring electrical responses in retinal photoreceptors using flash electroretinography ([Yan et al., 2017](#)). The neuroprotective function of Artemisinin against glutamate-induced oxidative stress was also reported by [Lin et al. \(2018\)](#). They investigated the effects of artemisinin on a mouse hippocampal cell line (HT-

22) damaged by glutamate-induced oxidative stress. The results demonstrated that the overproduction of ROS and the collapse of mitochondrial membrane potential caused by glutamate-induced oxidative stress could be rescued by the activation of the protein kinase B (Akt)/Bcl-2 signaling pathway in the cell line treated with artemisinin. In addition to artemisinin, administration of 3,5-diCQA, a phenolic compound extracted from *Artemisia argyi* H., prevented neuronal apoptosis from the mitochondrial pathway in the brain tissue of ICR mice exposed to TMT (Kang et al., 2016). In this study, 3,5-diCQA reduced TMT-induced mitochondrial ROS production and increased TMT-lowered mitochondrial membrane potential and ATP levels thus providing significant protection against TMT-induced mitochondrial dysfunction and cellular apoptosis (Kang et al., 2016).

Phosphorylation of microtubule-associated protein tau (p-tau) by protein kinase B/Akt (Akt), plays an essential role in Akt-mediated anti-apoptotic signaling (Ksiezak-Reding et al., 2003). Kang et al. (2016) also found that treatment with 3,5-diCQA could increase the ratio of phosphorylated-Akt (p-Akt)/Akt, which was reduced in the TMT-treated mice, and decrease levels of phosphorylated tau (p-tau) and Bax, which were increased in the TMT-treated mice. Taken together, *Artemisia* extracts exert their antioxidant and anti-apoptotic effects by decreasing mitochondrial ROS generation, increasing antioxidant enzyme levels, recovering mitochondrial membrane potential, and regulating signaling pathways such as

ERK/CREB and Akt/Bcl-2. These signaling pathways therefore could be potential targets for the treatment of epilepsy.

4.2 Anti-neuroinflammatory effects of the genus artemisia

Microglia-associated neuroinflammation is presumed to contribute to neuronal injury in various neurodegenerative disorders including epilepsy.

Neuroinflammation in epilepsy is primarily characterized by robust astrogliosis, microglial activation, and the production of cytokines and chemokines (McElroy et al., 2017; Vezzani, Balosso and Ravizza, 2019). Several bioactive molecules isolated from *Artemisia* extract were able to significantly eliminate neuroinflammation in brain tissue and neural cell lines. Artemisinin B isolated from *Aretmisia annua* and DSF-52 isolated from *Artemisia argyi* were found to exhibit significant anti-neuroinflammatory effects on LPS-activated BV2 cells (microglial cell model) (Zeng et al., 2014; Qiang et al., 2018). Both artemisinin B and DSF-52 significantly downregulated LPS-induced increase in NO production, and gene expression levels of inflammatory cytokines IL-1 β , IL-6, TNF- α , and upregulated gene expression levels of the anti-inflammatory cytokine IL-10 (Zeng et al., 2014; Qiang et al., 2018). DSF-52 also downregulated pro-inflammatory Prostaglandin E2 (PGE2), iNOS, COX-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) cytokines (Zeng et al., 2014). Another important

finding of their studies is that both artemisinin B and DSF-52 inhibited major inflammatory transcription factor NF- κ B in a dose-dependent manner (Zeng et al., 2014; Qiang et al., 2018). DSF-52 also inhibited the phosphorylation of NF- κ B, I κ B, and Akt, the activation and translocation of NF- κ B from the cytoplasm into the nucleus, and NF- κ B-DNA binding activity (Zeng et al., 2014). In addition to the Akt/I κ B/NF- κ B signaling pathway, DSF-52 also blocked JNK/p38 MAPKs and Jak2/Stat3 inflammatory signaling pathways by inhibiting the phosphorylation of respective molecules. Artemisinin B also reduced glial cell surface receptor TLR4 and its downstream adaptor protein MyD88 levels, which were abnormally high in the model group (Qiang et al., 2018). Both TLR4 and MyD88 are important because their association eventually leads to the activation of the NF- κ B and MAPK signaling pathways and thus to the expression of inflammatory cytokines and chemokines (Esen and Kielian, 2006). Based on these findings, the study suggests that artemisinin B inhibits neuroinflammation *via* the TLR4-MyD88-NF- κ B signaling pathway (Qiang et al., 2018). The ethanol extract of *Artemisia judaica* significantly reduced STZ-induced increase in the pro-inflammatory TNF- α and iNOS levels and expression in the cortical tissue of diabetic rats, comparable to the positive control metformin (Albasher et al., 2020). The study suggests that *Artemisia judaica* inhibits the development of cortical inflammation in diabetic rats (Albasher et al., 2020). In line with those findings, Artemisinin

isolated from *Artemisia annua* significantly reduced the immunoreactivity of glial cells, as detected by a decrease in GFAP and Iba1 markers, cleaved caspase 1, and IL-1 β levels, as well as restored microglia morphology in the cerebral cortex region of 3xTg AD mouse model ([Zhao et al., 2020](#)).

In addition to Artemesinin, the anticonvulsant effects of some other anti-inflammatory flavonoids isolated from the genus *Artemisia* have been confirmed in various experimental models of epilepsy, and their mechanisms of action have been explored. [Daneshkhah and Setorki. \(2019\)](#) showed that the anticonvulsant activity of essential oil (EO) of *Artemisia persica* significantly decreased seizure latency and tonic seizure in PTZ-kindled mice by reducing brain inflammation *via* downregulation of the expression of pro-inflammatory cytokines (IL-1 β and TNF- α). [Golechha et al. \(2011\)](#) observed that pretreatment of kainic acid (KA)-induced epileptic rats with naringin significantly attenuated seizures and cognitive deficits by inhibiting the production of the pro-inflammatory cytokine TNF- α in the brain. Hispidulin—a flavonoid compound found in *Artemisia abrotanum* L. and *Artemisia herba-alba*—substantially attenuated kainic acid-induced seizures and hippocampal neuronal cell death by the suppression of microglial activation and the production of pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α ([Lin et al., 2015](#)). [Zhu et al. \(2019\)](#) indicated that isoliquiritigenin pretreatment of kainic acid-induced epileptic rats has shown neuroprotective and

anti-inflammatory effects *via* the TLR4/MYD88 signaling pathway. These studies suggest that the genus *Artemisia* has antiepileptic therapeutic potential, at least in part because it contains flavonoid compounds with promising antioxidant and anti-inflammatory properties.

4.3 Anti-apoptotic and neuroprotective effects of the genus *artemisia*

Recently, several studies have documented the neuroprotective and antiapoptotic effects of *Artemisia*. As we mentioned in the previous section ([Section 4.1](#)), 3,5-diCQA isolated from *Artemisia argyi* demonstrated significant protection against neuronal apoptosis and cognitive function *via* the mitochondrial apoptotic pathway ([Kang et al., 2016](#)). [Kwon et al. \(2018\)](#) reported the neuroprotective effect of *Artemisia capillaris* and the possible involvement of apoptotic mechanisms. They showed that the ethanol extract of *A. capillaris* rescued BCCAO-induced neuronal degeneration and apoptosis, accompanied by an inhibition of BCCAO-induced increase of caspase-3-positive cells in the mouse hippocampus ([Kwon et al., 2018](#)). The ethanol extract of *A. judaica* increased the level of anti-apoptotic marker Bcl2 and decreased the level of pro-apoptotic marker Bax by blocking STZ injection-induced neuronal apoptosis in rat brains ([Albasher et al., 2020](#)).

Artemisinin isolated from *A. annua* was found to reduce p-tau levels and protect

brain tissue in 3xTg mice and SH-SY5Y cells *in vitro* ([Zhao et al., 2020](#)). In the brain tissue of 3xTg mice, Artemisinin treatment significantly reduced A β plaques in the cerebral cortex and hippocampus, damage to neurons and Nissl bodies, and the number of apoptotic cortical neurons. Further investigations indicated that p-ERK1/2, P-CREB levels, and Bcl-2/Bax ratio were significantly increased in the artemisinin-treated 3xTg animal group compared to the non-treated 3xTg group. In the SH-SY5Y cell line, pre-treatment with artemisinin prevented the A β (1–42)-induced SH-SY5Y cell death in a dose-dependent manner by protecting the mitochondrial membrane potential and decreasing cellular ROS production ([Zhao et al., 2020](#)). Moreover, artemisinin treatment resulted in a time- and dose-dependent increase in phosphorylated ERK1/2 and CREB levels in SH-SY5Y cells. Taken together, this study suggests that artemisinin exerts a potential anti-apoptotic effect on neural cells by regulating the ERK/p-CREB/Bcl2 pathway.

4.4 Beneficial effect of artemisia on cognitive function

Cognitive abnormality, especially impairment in learning and memory, is one of the serious comorbidities of epilepsy that has a significant negative impact on patients' quality of life ([Holmes, 2015](#)). Despite some reports, the mechanisms underlying epilepsy-associated cognitive impairment have not been completely elucidated. According to the literature, it is likely that many factors are involved,

including the dysregulation of neurotransmitter systems (cholinergic, glutamatergic, GABAergic), microglial activation and neuroinflammation, and oxidative stress, which ultimately result in neural tissue damage and cognitive impairment. Several *Artemisia* species (some of them mentioned in previous sections) have been identified as having neuroprotective potential and the capacity of restoring cognitive impairment by suppressing oxidative stress, neuroinflammation, and regulating the cholinergic neurotransmitter system. For instance, methanol extract of *Artemisia absinthium* significantly reduced cerebral infarct volume and short-term memory impairment in a mouse model of cerebral ischemia induced by middle cerebral artery occlusion (MCAO) *via* regulation of oxidative stress/antioxidant parameters (Bora and Sharma, 2010). In this study, *Artemisia* treatment resumed levels of antioxidant enzymes (GSH, SOD, and CAT) that were decreased in the brain of the MCAO model, and the concentration of the thiobarbituric acid reactive substance (TBARS)—a lipid peroxidation marker—that was increased in the brain of MCAO model (Bora and Sharma, 2010). Interestingly, administration of the plant extract before focal cerebral ischemia also prevented short-term memory impairment suggesting that *A. absinthium* can not only restore already damaged neural tissue but also protect them from damage (Bora and Sharma, 2010). In another study using a mouse model of Alzheimer's disease established by intra-cerebroventricular injection of the toxic fragment of

A β (A β 25-35), artemisinin B—a type of artemisinins derived from *A. annua*—significantly ameliorated learning and memory of this mouse model in water-maze and step-through tasks. In the same model group, artemisinin B also inhibited the activation of microglia as well as the loss of the Nissl bodies and synaptophysin in the CA1 neurons of the hippocampus (Qiang et al., 2018). These studies indicated that oxidative stress, microglial activation, and neuroinflammation are involved in neuronal damage in the hippocampus, and *Artemisia* extracts could protect and restore memory deficiency by suppressing those processes. The genus *Artemisia* species can also restore cognitive impairment by modulating neurotransmitter and neuromodulator activity. 3,5-diCQA pretreatment significantly inhibited TMT-induced disruption of the cholinergic neurotransmission and cognitive impairment by increasing acetylcholine (ACh) levels and decreasing acetylcholinesterase (AChE) activity in the brain tissue of ICR mice compared to negative controls (Kang et al., 2016). In line with these findings, Kwon et al. (2018) reported that treatment with ethanol extracts of *A. capillaries* could recover BCCAO-mediated neurodegeneration and cognitive impairment in mice by stimulating nicotinic acetylcholine receptors (nAChRs) and inhibiting AChE activity. They showed that the nonselective neuronal nicotinic receptor antagonist mecamylamine markedly blocked the neuroprotective effect of *A. capillaris* (Kwon et al., 2018). The authors hypothesize that the plant metabolites might activate the PI3K-Akt signaling

cascade, which leads to the activation of neuronal nAChRs (Kwon et al., 2018).

Hence, the study suggests that the neuroprotective and pro-cognitive action of *A. capillaris* is due to the activation of the cholinergic neurotransmitter system in the brain.

These data altogether conclude that MOS, mitochondrial dysfunction, neuroinflammation, disbalanced neurotransmission, and ultimately neuronal death/apoptosis could be also implicated in epileptogenesis and cognitive impairment. We assume that targeting these processes using Artemisia extracts have promising protective roles against the seizure generation, epileptogenesis, and cognitive comorbidities of epilepsy (Figure 2).

ROS production and neuroinflammation as well as regulating disbalanced ...

Epilepsy is defined as one of the World Health Organization (WHO) public health priorities for its effective care, prevention, and treatment. It is a potentially life-threatening disease and associated with increased disability and mortality (Thurman et al., 2018). Although AEDs are the primary treatment method for controlling epilepsy, most of these drugs are considered to be associated with temporary seizure control, drug resistance, and cognitive abnormalities (Sørensen and Kokaia, 2013). Growing evidence reveals that oxidative stress, neuroinflammation, and dysregulated neurotransmission contribute to the

pathophysiology of epilepsy and cognitive impairment. Neuronal damage and death/apoptosis are most likely to be directly or indirectly caused by neuronal hyperexcitability, oxidative stress, and neuroinflammation. Therefore, any antioxidant, anti-inflammatory, and neuroprotective interventions might prevent epileptogenesis and its cognitive comorbidities.

Nowadays, the growing need for more natural sources of medicine and certain prominent pharmacological activities of Artemisinin has driven scientific interest in *Artemisia* species. In recent years, a series of experimental studies have demonstrated that certain genus *Artemisia* has anticonvulsant effects in animal models of epilepsy *in vivo* (Table 1) and antioxidant, anti-neuroinflammatory, and neuroprotective effects in neural cell lines *in vitro* (Tables 2, 3, 3), in which oxidative stress were induced and epileptic seizures were developed.

Antioxidant, anti-inflammatory, and neuroprotective effects of *Artemisia* species *in vivo* and its mechanisms of action.

in vitro and its mechanisms of action.

However, the exact pharmacologically active compounds of *Artemisia* (except artemisinin) and the underlying mechanisms through which those bioactive compounds exert antiepileptic effects have not been extensively investigated.

Based on all the data included and analyzed in the review, we assume that genus

Artemisia subspecies possess potential antiepileptic and neuroprotective properties thanks to their antioxidant, anti-inflammatory, neurotransmitter regulating, and anti-apoptotic activities. The bioactive secondary metabolites of the genus Artemisia plant are likely to act through modulating multiple signaling pathways, such as extracellular regulated protein kinases (ERK/CREB/Bcl-2) pathway and intracellular (mitochondrial) pathway, as well as an emerging nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway.

Therefore, the use of natural bioactive compounds isolated from Artemisia as potential pharmacological modulators targeting the aforementioned signaling pathways might be an alternative option for the treatment of epilepsy and its cognitive comorbidities. However, although many experimental studies have demonstrated anti-epileptic and neuroprotective activities of Artemisia species, most of them have emphasized crude plant extracts or fractions, and only a few pure bioactive compounds have been used in the studies (see [Tables 1–3](#)). In addition, there are other pitfalls in using Artemisia: long-term and low-dose Artemisia exposure may induce free radical scavengers that disrupt the endoperoxide bridge structure of Artemisia. Moreover, unexpected metabolic dysfunction or other abnormalities may occur after excessive use of Artemisia, including neurotoxicity and/or sperm DNA damage-induced genotoxicity ([Singh, Giri and Giri, 2015](#); [Sun and Zhou, 2017](#)) Therefore, to ensure safety at appropriate

doses, long-term testing should be evaluated. Finally, there is currently no preclinical and clinical trial evidence showing that *Artemisia* has a therapeutic effect on epilepsy.

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Chapter7

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease associated with motor neurons (MNs) degeneration, muscle weakness, fasciculations, muscle atrophy, swallowing and speech disabilities, paralysis and finally death (van Es et al., 2017). Death is usually caused by respiratory failure and occurs in 2–4 years after the onset (Huisman et al., 2011; Wittie et al., 2013; Oskarsson et al., 2018). ALS occurs with an incidence of 1–2 cases in 100,000 individuals per year, with about 90% of cases being sporadic and 10% characterized as familial (Oskarsson et al., 2018). More than 20 genes have been associated with familial ALS, of which the ones encoding for chromosome nine open reading frame 72 (C9orf72), superoxide dismutase 1 (SOD1), Fused in sarcoma (FUS), and TAR DNA-binding protein 43 (TDP-43), account for most of the cases (Chia et al., 2018).

However, despite decades of research, the mechanisms underlying ALS pathogenesis remain unclear. ALS appears as a multifactorial disease where several processes operate simultaneously. These include oxidative stress, glutamate excitotoxicity, mitochondrial dysfunction, inflammatory response, impairment of

axonal transport, impaired protein homeostasis and transcriptional dysregulation (Giribaldi et al., 2013; Mejzini et al., 2019; Bendotti et al., 2020; De Marchi et al., 2021).

Epidemiological studies and clinical observations have shown evidence of sexual dimorphism in ALS (Vegeto et al., 2020). Men display higher risk of developing ALS, with a male/female ratio reported between 1 and 3, depending on geographic area and population considered (Manjaly et al., 2010). Although the overall survival is similar in both sexes, the disease appears earlier in men (Blasco et al., 2012). Moreover, the ALS phenotype is different in males and females, with a predominance of limb onset in men and bulbar onset in women (Blasco et al., 2012).

Currently, no cure is available for ALS and the molecules tested, alone or in combinations, in animal models and in patients did not lead to real improvements (Mitsumoto et al., 2014; Wobst et al., 2020). The only therapeutic drugs approved for ALS treatment are riluzole, a glutamate receptor antagonist approved in 1995, and edavarone, a free radical scavenger approved by the FDA in 2017 (Oskarsson et al., 2018; Yoshino 2019; Wobst et al., 2020). Riluzole has been shown in clinical trials to prolong median survival from 11.8 to 14.8 months, postponing the use of surrogate approaches, such as tracheotomy and mechanical ventilation

([Lacomblez et al., 1996](#); [Miller et al., 2012](#)). Edavarone modestly slows the rate of disease progression and prolongs the tracheostomy-free survival in ALS patients ([Writing Group and Edaravone \(MCI-186\) ALS 19 Study Group. 2017](#); [Okada et al., 2018](#)).

The pathogenic role of anomalous acetylation of NF- κ B/RelA and histones in ALS

In the central nervous system (CNS), the nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) transcription factors play a pivotal role in a number of physiological processes including neurogenesis ([Zhang and Hu, 2012](#)), neuritogenesis ([Gutierrez and Davies, 2011](#)), learning and memory ([Kaltschmidt and Kaltschmidt, 2015](#)). On the other hand, NF- κ B dysregulation has been associated to neurodegenerative mechanisms occurring in pathological conditions such as stroke, epilepsy, Parkinson's disease (PD) and Alzheimer's disease (AD) ([Srinivasan and Lahiri, 2015](#); [Bellucci et al., 2020](#)).

The NF- κ B family is composed by five different members [p50/p105 (NF- κ B1), p52/p100 (NF- κ B2), p65 (RelA), c-Rel and RelB] that combine to form the transcriptionally active dimer ([Gilmore and Wolenski 2012](#); [Lanzillotta et al., 2015](#)). Neurotoxic stimuli, including ischemia ([Inta et al., 2006](#)), glutamate ([Pizzi](#)

et al., 2002), β -amyloid ([Pizzi et al., 2005](#)), or 1-methyl-4-phenylpyridinium (MPP+) ([Ghosh et al., 2007](#); [Sarnico et al., 2008](#)) induce the activation of p50/RelA dimer and the transcription of pro-apoptotic factors, such as Bim and Noxa. The opposite effects exerted by the NF- κ B dimer p50/RelA on neuron survival can rely on changes in the acetylation state of RelA within the nuclear p50/RelA ([Lanzillotta et al., 2010](#); [Sarnico et al., 2012](#)). In particular, a specific pro-apoptotic acetylation profile of nuclear RelA, involving the general deacetylation of lysines but a site-specific acetylation at the lysine 310 [acRelA(K310)], occurs in lethal ischemia but not in protective preconditioning brain ischemia ([Lanzillotta et al., 2010](#)). The correction of RelA acetylation by pharmacological HDAC modulation (see below) reduced both the brain infarct volume and the neurological deficits ([Lanzillotta et al., 2013](#); [Faggi et al., 2018](#)).

A pathogenic role for the activation of NF- κ B/RelA has been suggested also for ALS.

Major genetic risk factors linked to ALS, including mutations in genes encoding for SOD1, OPTN, TBK1, TDP-43 and FUS, activate the NF- κ B pathway ([Källstig et al., 2021](#)). NF- κ B/RelA levels were elevated in mutant SOD1 MNs and astrocytes cellular model of ALS ([Prell et al., 2014](#); [Ikiz et al., 2015](#); [Yin et al., 2018](#)). Interestingly, Yin and colleagues reported that the MNs vulnerability to the

conditioned medium obtained from mutant SOD1 astrocytes was dependent on the activation of the phosphorylated form of RelA, a modification known to promote RelA acetylation at the K310 residue ([Yin et al., 2018](#)). NF- κ B/RelA was increased with disease progression in the SOD1(G93A) and TDP-43 mouse models of ALS ([Swarup et al., 2011](#); [Frakes et al., 2014](#)). Neuron-specific expression of super-repressor form of the inhibitory κ B proteins (IkB-SR) ameliorated behavioral and pathologic phenotypes in three mouse models of ALS carrying either human mutated TDP-43 or SOD1 transgenes ([Dutta et al., 2020](#)). Furthermore, NF- κ B inhibition by administration of withaferin A alleviated disease symptoms in TDP-43 mice ([Swarup et al., 2011](#)) and extended lifespan of SOD1(G93A) and SOD1(G37R) mouse models of ALS ([Frakes et al., 2014](#); [Patel et al., 2015](#)). In addition, levels of NF- κ B/RelA were higher also in the spinal cord of ALS patients when compared with age-matched healthy subjects ([Jiang et al., 2005](#); [Swarup et al., 2011](#)). Notably, as previously found in ischemic brain neurons ([Lanzillotta et al., 2010](#); [Lanzillotta et al., 2013](#)), the pro-apoptotic acetylation profile acRelA(K310) was observed also in the lumbar spinal cord of SOD1(G93A) mice ([Schiaffino et al., 2018](#); [Bankole et al., 2022](#)). These results suggest that NF- κ B signaling may represent a unique therapeutic target for ALS disease. The beneficial effect of a therapeutic approach inhibiting NF- κ B/RelA could be enormously enhanced by switching-off the p50/RelA pro-inflammatory/pro-apoptotic activity

without modifying the dimer effects on neurogenesis, neuritogenesis and cell survival.

Histone acetylation at lysine residues is an important epigenetic mechanism regulating chromatin folding and the accessibility of transcription factors to their target genes (Eberharter and Becker 2002). Altered histone acetylation has been associated with reduced neuronal survival and pathological CNS conditions, including stroke, PD, AD and Huntington's disease (Konsoula and Barile, 2012; Uzdensky and Demyanenko 2021). A growing body of evidence suggests a possible role for a dysregulation of epigenetic mechanisms, including histone acetylation, also in the occurrence and progression of ALS (Jimenez-Pacheco et al., 2017; Bennett et al., 2019; Zhang et al., 2022). For example, overexpression of FUS or TDP-43 in yeast ALS proteinopathy models resulted in histone hypo- and hyperacetylation, respectively, suggesting that each proteinopathy may correspond to a specific alteration of histone acetylation (Chen K et al., 2018). Moreover, histone acetylation was reduced in the spinal cord of SOD1(G93A) and Tg FUS^{+/+} mouse models of ALS (Schiaffino et al., 2018; Rossaert et al., 2019; Bankole et al., 2022). In light of these considerations, histone acetylation appears as a potential target for ALS treatment.

The acetylation state of NF- κ B/RelA and histones results from the opposing activity of histones acetyltransferases (HATs) and histone deacetylases (HDACs).

Until now, eighteen mammalian HDACs have been characterized and grouped into four major classes according to their homology with yeast HDACs (Shukla and Tekwani, 2020). The Class I, II, and IV are known as classical HDACs and use zinc as cofactor. Class III HDACs, commonly known as sirtuins (SIRT 1–7), are NAD dependent and are involved in regulation of metabolism, stress and aging. Members of class I HDAC (HDAC 1, 2, 3 and 8) are most involved in the regulation of acetylation state of NF- κ B/RelA (Chen and Greene, 2004). A body of evidence showed an unbalance of HATs and HDACs activity in patients as well as in preclinical models of ALS (Schmalbach and Petri, 2010; Lazo-Gómez et al., 2013; Jimenez-Pacheco et al., 2017; Shukla and Tekwani, 2020; Klingl et al., 2021). HDAC1 silencing or treatment with pan-HDAC inhibitors exert a protective role against wild-type or pathological mutant TDP-43 toxicity, suggesting TDP-43 acetylation as a new potential therapeutic target (Sanna et al., 2020). While data from expression analysis of HDAC isoforms in post-mortem brain and spinal cord tissue of ALS patients remain controversial (Janssen et al., 2010; Dios et al., 2019), recent findings suggest that increasing HDACs activity might exert a protective role in ALS. This is the case of the class IIa HDAC 4, whose expression in skeletal

muscle of an ALS mouse model is responsible for compensatory reinnervation ([Pigna et al., 2019](#)).

The anomalous acetylation of NF- κ B/RelA and histones can be corrected by the association of resveratrol and HDAC inhibitors

We reported that the pathological acetylation profile of RelA and histones in brain ischemia can be corrected by the synergistic combination of low doses of the epigenetic drugs resveratrol and the HDAC inhibitor MS-275 (entinostat) ([Lanzillotta et al., 2013](#)).

Resveratrol is a polyphenol stilbene widely investigated for the prevention or treatment of different diseases thanks to its anti-aging, anti-inflammatory, anti-oxidant and anti-tumorigenic properties ([Rauf et al., 2017](#); [Parrella et al., 2020](#); [Zhang et al., 2021](#)). Among its multiple mechanisms of action, the molecule is able to activate the class III NAD⁺-dependent HDAC SIRT1 ([Lee et al., 2019](#)) and AMP-activated protein kinase (AMPK), a serine–threonine kinase acting as a key metabolic and stress sensor/effector ([Ruderman et al., 2010](#)).

The synthetic benzamide MS-275 has been shown to inhibit class I HDAC (HDAC 1–3) with excellent pharmacokinetic properties ([Simonini et al., 2006](#); [Khan et al., 2008](#)). The molecule is in clinical trials for the treatment of different types of cancer ([Wang et al., 2022](#)).

The use of resveratrol and MS-275 promoted a synergistic neuroprotection in primary cortical neurons exposed to oxygen and glucose deprivation (OGD) and in mice subjected to transient middle cerebral artery occlusion (tMCAO) ([Lanzillotta et al., 2013](#)). Similarly, a single treatment with resveratrol and MS-275 reduced stroke-mediated brain injury and inflammation in mice subjected to permanent MCAO ([Mota et al., 2020](#)).

The beneficial effects of the combination of resveratrol and MS-275 are mediated by the reversion of the mismatch of RelA acetylation state by respectively reducing the acetylation at the K310 *via* SIRT1 activation and enhancing the RelA general acetylation ([Lanzillotta et al., 2013](#)). The drug combination also reverted the histone H3 deacetylation produced by the ischemic injury ([Lanzillotta et al., 2013](#)). Of note, the drug effect was sustained by the resveratrol-promoted AMPK activation that, by increasing generation of acetyl-CoA, can support HAT activity ([Lanzillotta et al., 2013](#)). Moreover, AMPK could also corroborate SIRT1

activation by resveratrol *via* induction of NAD⁺, the fundamental co-factor for class III HDACs (Ruderman et al., 2010).

The substitution of MS-275 with valproate (VPA), an antiepileptic/mood stabilizer endowed with inhibitory activity for class I and class IIa HDACs (Perucca, 2002; Gurvich et al., 2004), in association with resveratrol exerted synergistic neuroprotection in the OGD cellular model of brain ischemia, by correcting the pathological acetylation state of RelA and reverting the histone H3 deacetylation (Faggi et al., 2018). Moreover, a single intraperitoneal administration of the association of resveratrol and VPA synergistically reduced infarct volume and neurological deficits in the tMCAO mouse model of ischemic stroke (Faggi et al., 2018). Interestingly, VPA increased RelA general acetylation *in vivo* (Chen S et al., 2018).

Resveratrol and many HDAC inhibitors have been individually studied also against ALS (Chuang et al., 2009; Carrera-Juliá et al., 2020; Shukla and Tekwani, 2020; Klingl et al., 2021; Novak et al., 2021). The major effects of resveratrol and the main HDAC inhibitors tested individually or in combination in ALS preclinical models or patients are reported in Table 1 and described in the following sections.

Chapter8

Effect of resveratrol in ALS preclinical models and patients

A protective effect of resveratrol in ALS was demonstrated in neuronal cell lines expressing the SOD1(G93A) mutant (Kim et al., 2007; Barber et al., 2009; Wang et al., 2011). Interestingly, it has been reported that bone marrow mesenchymal stem cells from ALS patients displayed down-regulation of AMPK/SIRT1 signalling, which was rescued by treatment with resveratrol (Yun et al., 2019). In addition, resveratrol prevented the neurotoxic effect of cerebrospinal fluid (CSF) from ALS patients on cultured MNs (Yáñez et al., 2011).

Dietary treatment of SOD1(G93A) male and female mice with resveratrol (160 mg/kg/day) delayed disease onset, extended lifespan of approximately 10%, and preserved MNs survival (Mancuso et al., 2014). Interestingly, resveratrol delayed disease onset in a sexually dimorphic fashion, postponing symptoms onset of 2 weeks in males and 1 week in females (corresponding to a delay of approximately 15% and 11%, respectively).

Another study reported that dietary resveratrol at the dose 25 mg/kg/day did not promote functional effects in SOD1(G93A) female mice (Markert et al., 2010). Conversely, Han and colleagues reported that intraperitoneal administration of resveratrol at the dose 20 mg/kg twice a week delayed disease onset, extended survival of 7% and reduced MNs loss in SOD1(G93A) male mice (Han et al., 2012).

Effect of HDAC inhibitors in ALS preclinical models and patients

Intraperitoneal treatment of SOD1(G93A) male mice with 1 mg/kg/day of trichostatin A (TSA), a pan-HDAC inhibitor of all zinc-dependent HDACs (Khan et al., 2008), promoted an increase of 7% in lifespan, together with a reduction of MNs loss, gliosis, muscular atrophy and neuromuscular junction denervation (Yoo

and Ko, 2011). However, studies in human lymphoblasts *in vitro* pointed out genotoxic effects of TSA, raising doubts about a possible clinical use of the molecule (Olaharski et al., 2006).

Sodium phenylbutyrate (NaPB) is an inhibitor of HDAC class I and IIa (Kusaczuk et al., 2015). A recent study has shown the beneficial effect of NaPB in improving mitochondrial bioenergetics in a cellular model of ALS (Li et al., 2022). NaPB injected intraperitoneally at the dose of 400 mg/kg/day, prolonged the survival by 21%, ameliorated motor function and promoted expression of anti-apoptotic genes in SOD1(G93A) male mice (Ryu et al., 2005). The effect of NaPB on SOD1(G93A) mice was confirmed in another study, where the molecule injected intraperitoneally at the same dose significantly increased motor function, extended survival by 13% and attenuated MNs loss (Petri et al., 2006). In a third study, daily intraperitoneal treatment of SOD1(G93A) male mice with NaPB at the dose 300 mg/kg improved survival by approximately 13% while ameliorating body weight loss and grip strength (Del Signore et al., 2009). A phase II clinical trial studying the effect of an increasing dosage of NaPB over 12 weeks to a maximum of 21 g/day, reported the safety and tolerability of the drug, but did not evaluate its efficacy in ALS (Cudkowicz et al., 2009).

ACY-738 is a novel HDAC inhibitor selective for HDACs 1, 2, 3 and 6 (Mithraprabhu et al., 2013; Jochems et al., 2014). Treatment with ACY-738 rescued axonal transport deficits in MNs expressing FUS mutation derived from induced pluripotent stem cells (iPSCs) from ALS patients (Guo et al., 2017). Recently Van Den Bosch and colleagues have shown that the oral treatment of the ALS transgenic mouse model Tg FUS^{+/+} (both males and females) with ACY-738 (100 mg/kg) slowed down the disease progression, and improved the lifespan (Rossaert et al., 2019). Interestingly, the authors reported a larger survival extension in males (76% in males, 24% in females) (Rossaert et al., 2019). The beneficial effect of the molecule was associated with a mitigation of lipid metabolism alterations and a restoration of global histone acetylation, but not with a reduction of astrocytosis nor microgliosis (Rossaert et al., 2019; Burg et al., 2021).

Tubastatin A is a highly selective inhibitor of HDAC 6 (Butler et al., 2010) which has been investigated in different neurological disease animal models (Shen et al., 2020). Similarly to ACY-738, pharmacological inhibition of HDAC 6 by tubastatin A reverted axonal transport deficits in ALS patient-derived MNs with mutations for FUS and TDP-43 (Guo et al., 2017; Fazal et al., 2021).

MC1569 is a novel selective inhibitor of the HDAC class II (Mai et al., 2005).

Treatment of SOD1(G93A) male and female mice with MC1568 (60 mg/kg/day, i.p.) restored glutamate uptake capacity in spinal cord, but did not increase lifespan (Lapucci et al., 2017). In a second study, i.p. treatment of SOD1(G93A) male and female mice with the drug at 40 mg/kg/day promoted early improvement of motor performances that disappeared at later stages of disease (Buonvicino et al., 2018). The transient motor improvement was coupled with increased skeletal muscle electrical potentials and muscle expression of myogenic genes, but not with a protection of MNs from neurodegeneration (Buonvicino et al., 2018). No evidence of sex-specific effect was found.

VPA treatment reduced neurotoxicity in motor neuron cellular models of ALS (Ragancokova et al., 2010; Jiang et al., 2016; Gyawali et al., 2022). *In vivo*, oral administration of VPA at the antiepileptic dose of approximately 500 mg/kg/day increased lifespan by 8% without delaying the disease onset in SOD1(G93A) male mice (Sugai et al., 2004). Feng and colleagues reported that VPA treatment (300 mg/kg twice a day, i.p.) in SOD1(G93A) male and female mice delayed motor deficits onset by 8%, improved lifespan by 10%, and had beneficial effects on motor dysfunction (Feng et al., 2008). In another study, oral treatment of SOD1(G93A) male and female mice with the drug at antiepileptic dose slowed down MNs loss without significantly improving lifespan (Crochemore et al.,

2009). VPA, when administered intraperitoneally at the antiepileptic dose of 250 mg/kg/day to SOD1(G86R) male mice, delayed the disease onset of 10%, but failed in improving mean survival (Rouaux et al., 2007). VPA efficacy has been investigated also in phase II clinical trials for ALS, but VPA-treated subjects (1,500 mg daily) did not show a difference in survival or disease progression rate compared to placebo-treated patients (Piepers et al., 2006).

The association of resveratrol and HDAC inhibitors in the ALS treatment

It has been shown the resveratrol and HDAC inhibitors at very low doses can synergize in promoting neuroprotection in the SOD1(G93A) mouse model of ALS (Schiaffino et al., 2018; Bankole et al., 2022).

When administered to combined male and female SOD1(G93A) mice, the association resveratrol (136 µg/kg/day) and MS-275 (4 µg/kg/day) delayed symptoms' onset by 3 weeks and prolonged lifespan by 2 weeks, corresponding to an increase of 25% and 12%, respectively (Schiaffino et al., 2018). Furthermore, the treatment rescued MNs, and increased the levels of anti-apoptotic B-cell lymphoma-extra large (Bcl-xL) and neurotrophic Brain-Derived Neurotrophic

Factor (BDNF) in the lumbar spinal cord, without modifying microglia activation (Schiaffino et al., 2018).

In a similar fashion, the treatment of SOD1(G93A) male and female mice with the association resveratrol (136 µg/kg/day) and VPA (40 µg/kg/day) promoted a significant improvement in motor performances, the delay of disease onset, and longer survival (Bankole et al., 2022). Moreover, the epigenetic drugs protected MNs from neurodegeneration, reduced immunoreactivity of microglia, and increased expression of Bcl-xL and BDNF levels in the lumbar spinal cord (Bankole et al., 2022).

In accordance with studies on brain ischemia models (Lanzillotta et al., 2013; Faggi et al., 2018), the beneficial effects promoted by the association of resveratrol and HDAC inhibitors was coupled to the rescue of RelA and the histone 3 acetylation state, and of AMPK activation (Schiaffino et al., 2018; Bankole et al., 2022), indicating a mechanism of action based on the reversion of the mismatch of RelA and histone acetylation also in ALS.

It is important to note that resveratrol can also modify the acetylation status of other proteins potentially involved in ALS pathogenesis. For example, resveratrol was able to deacetylate p53 and the peroxisome proliferator-activated receptor gamma coactivator1alpha (PGC1- α) in preclinical models of ALS (Kim et al.,

2007; Mancuso et al., 2014). Both the proteins have been involved in mechanisms of MNs death (Ranganathan and Bowser, 2010; Lazo-Gómez et al., 2013), and their deacetylation has been associated with neuroprotection (Hasegawa and Yoshikawa, 2008; Panes et al., 2022). Therefore, it can be speculated that other mechanisms, besides the modulation of RelA and histone acetylation, may support the beneficial action of this pharmacological association.

Figure 1 depicts the mechanisms responsible for NF- κ B/RelA and H3 histone acetylation upon treatment with combination of resveratrol and HDAC inhibitors in ALS mice.

the SOD1(G93A) ALS mouse model, NF- κ B/RelA is present in its aberrantly ...

The SOD1(G93A) mouse model displayed a sexually dimorphic behavior in response to the association of resveratrol and VPA (Bankole et al., 2022).

Specifically, in accordance with a previous study investigating the effect of resveratrol in SOD1(G93A) mice (Mancuso et al., 2014), males showed positive outcomes in the early phases of the disease (onset delay of 27%). Conversely, only in females the epigenetic drugs reduced motor deficits in a later phase of the disease and prolonged survival by 6%. These findings suggest a possible action of resveratrol and VPA on specific sex-related molecular targets. For example, it is plausible that these compounds could potentiate the neuroprotective effect of

female sex steroids *in vivo* ([Bankole et al., 2022](#)). In support of this, both resveratrol and VPA are endowed with estrogenic properties ([Stempin et al., 2013](#); [Qasem, 2020](#)).

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Chapter9

Social relationships are important for social development and long-term psychological health. These relationships are maintained by behaviors that are

tightly linked with various emotions and affective states, which are themselves defined by social and other environmental contexts (Spoor and Kelly, 2004; de Waal, 2011). In the past several decades, the interest in applying functional brain imaging for the study of human emotion has grown considerably (Davidson and Irwin, 1999; Phan et al., 2004; Wager et al., 2008). However, the application of these same methods to study emotion and affect in non-human animals (hereafter animals) is still early in its development.

Recent advances in the field of brain imaging have increasingly allowed for direct observation of neural processes active during presumed emotionally evocative events, particularly in humans, as illustrated throughout this review. In both humans and animals, imaging allows for the relatively non-invasive, longitudinal study of neural processes; the reduced invasiveness of imaging techniques also allows one to study typically non-researched animals (e.g., pets and zoo animals). In particular, positron emission tomography (PET) imaging allows uptake of a radiotracer while an animal is unrestrained, awake, and potentially engaged in ethologically and socially relevant tasks. While imaging does not currently allow us to examine activity at the level of the neuron, it allows for more naturalistic social interactions that other methods (e.g., electrophysiological recording) do not. Another drawback of imaging methods is that they are still indirect measures (e.g., with the use of PET scan, we only measure glucose uptake in the neuron as a proxy

for neuron activity) and cannot be used for all behaviors for both animals and humans (e.g., in an fMRI scanner, subjects have to stay immobile, and it also requires to create a stimulus that the subject can easily watch or imagine when laying in the scanner). Despite these limitations, imaging methods are powerful tools and have led to significant advances in the field of behavioral neuroscience. While the study of animals in imaging studies could significantly improve our understanding of the physiological substrates of affective processes, few imaging studies have focused on animals.

Emotion is not easily defined, and several definitions have been put forward (for a recent review, see Paul and Mendl, 2018). The term “affect,” which is a closely related term to emotion, tends to be used interchangeably with the term *emotion* by some authors, although others make a clear distinction between the two terms (Russell, 2003; Shouse, 2005). It has been suggested that an emotion is a multicomponent response (subjective, physiological, neural, and cognitive) to the presentation of a stimulus or event (Paul and Mendl, 2018). Other authors posit that an emotion is, perhaps more broadly, a state of the nervous system that is provoked by extrinsic or intrinsic stimuli (Anderson and Adolphs, 2014).

Alternatively, “affect” is often used as an umbrella-term or “superordinate category” to describe, at the most basic level, a number of related constructs including emotions, emotion episodes, mood, dispositional states, and traits, which

are themselves largely distinguished from one another according to their durations (Gross, 1998). The concept of affect is also often at the center of the definition of emotional episodes, as a state that is positively or negatively valenced and either activated or deactivated (Russell, 2003; Bliss-Moreau, 2017). We do not seek to debate these definitions, but to promote clarity in this review we offer a classic, broad, functional definition for use in this discussion; i.e., emotions are temporary and relatively brief affective states that result from internal and external stimuli that are situated within social and environmental contexts; and emotions are reflected in changes in the brain and periphery of the body that serve to facilitate a locally rational reaction to those stimuli and motivate action. Emotions are frequently elicited by the expectancy of a reward or a punishment, thus it is also possible to classify emotions into categories of responses to rewarding (i.e., positive) or punishing (i.e., negative) stimuli (Mendl et al., 2010). Additionally, although we define emotions as a relatively brief state, they can occur repeatedly and can also be studied in the long term, for example throughout the duration of social relationships (e.g., in love, or grief), where relationships influence emotion expression for long periods and can have lasting effect on physiology and neurobiology (for example on the dopamine system where attachment is associated with a higher D1 binding in the brain; Hostetler et al., 2017).

A recent review provides a conceptual framework and an exhaustive list of the various methods used to study emotions in animals and further illustrates how complex it is to understand them (Kremer et al., 2020). They suggest that the affective and emotional states of animals can be assessed by four components of an affective episode: (1) The feeling or subjective component is described as a psychological construct that may not be shared by all animals and which is impossible to assess in non-talking animals. (2) The behavioral component is described as a change in behavior that may be a cause or a consequence of emotion depending on the authors (Anderson and Adolphs, 2014) and specific behaviors have been associated with positive and negative emotions and can be quantified to assess the emotional state (positive: e.g., play behavior, anticipatory behaviors, consumptive behaviors, affiliative behaviors; negative: e.g., freezing, aggressive behaviors, displacement behaviors). (3) The cognitive component is described as a source of *cognitive bias* during cognitive tests. It is further described as the result of the bidirectional link between emotional and cognitive processes (judgment, attention, and memory) (Harding et al., 2004; Mendl et al., 2010). (4) The physiological component is described as the observed change in physiological systems (e.g., the neuroendocrine, immune, and autonomic nervous systems) during an affective experience. When assayed individually, any of these four affective components should be interpreted with restraint. When studied in

animals, without the potential to directly communicate the psychological component of an affective experience, it may be more difficult to conclude that a change in either behavior, cognition or physiology alone is a result of a shift in affect. Thus, recent studies on animal emotion and affect try to combine several measures of behavior, cognition, and/or physiology; and they often combine multiple components of affect (e.g., physiology *and* behavior) into consideration (Maninger et al., 2017b; Cook et al., 2018; Kato et al., 2018).

Other frameworks exist in contrast to the “classical” view of emotions, for example the “Theories of Constructed Emotion” or TCE (Bliss-Moreau, 2017; Feldman Barrett, 2017), which states, for example, that discrete emotions cannot be consistently attributed to specific brain networks. For example, they would suggest that there is no specific “fear circuit” or “anger circuit.” Rather, the TCE would suggest that discrete emotions are higher order interpretations of more basal and diffuse “affect” that is contextualized within the current social situation. Various elements of affect would themselves be constructed of several interacting brain networks. In this framework, “affect,” when defined as a perturbation in allostasis, is the principal description of emotion, varying across two axes: arousal (high to low) and valence (positive to negative). Affect, combined with context, previously lived experience, and (potentially) the ability to conceptualize, results in emotion. Emotions experienced by animals may or may not be recognizable to humans; but

importantly, they did not evolve as modules recognizable by a set of clearly identifiable brain areas or networks if we refer to the TCE. However, in animals as in humans, we are capable of studying biological and behavioral responses to affective stimuli.

Emotions in social relationships, what we will call “social emotions,” are of major importance in animal societies (Spoor and Kelly, 2004). Social emotions are defined by the social context in which they occur (Panksepp, 1998), and which exist as dependent upon the affect, behavior, or cognition of others. Social emotions may be considered adaptive, since they allow for the creation and maintenance of valuable relationships which may subsequently provide fitness benefits (Spoor and Kelly, 2004). Stable relationships are of major importance for animals (including humans) that rely on conspecifics for survival and success, as they provide health benefits and lower mortality risks (House et al., 1988). The frequency of affiliative behaviors that an individual displays with other members of their social group is associated with increased reproductive success in several species (Brent et al., 2014b). Social bonds and associated social emotions may also seem to be accompanied by several “drawbacks,” especially when they are negative in valence, i.e., associated with punishment (distress, pain), as in the cases of loneliness and grief. One might wonder if these negative emotions are adaptive and how they evolved. Here, we posit that negative emotions should not be viewed

as “maladaptive” and, to the contrary, may themselves have an adaptive role (Nesse, 1990). Some evidence suggests that during separation, titi monkeys display behavior (such as lip smacking, an affiliative behavior in this species) that may strengthen the valuable relationship (Maninger et al., 2017b). Another (also potentially positive for reproduction) example is seen in jealous macaques, where males react with aggressive behaviors toward their male rival (Rilling et al., 2004), perhaps as an attempt to disrupt the formation of a relationship between his current consort and a potential competitor.

Thus, social emotions seem to be shared across animal species, and there is growing evidence that shared neural and hormonal substrates are involved in the expression of affect and emotion across species as a result of a common evolutionary history (Panksepp, 2011). One approach to understanding animal emotion is through the comparative method (Nesse, 1990), in which human and animal emotional experiences are compared and contrasted. The comparative approach operates on the understanding that because vertebrates share common neural structures, and especially humans with other primates (Hori et al., 2021), they may share the products of those common structures, for example emotions or affective states (Panksepp, 2011). Even if direct comparison between distant species (including birds, fish, and amphibians) is a very delicate operation, some brain areas have been clearly identified as homologous, such as the lateral septum

(LS) ([Goodson, 2005](#)) periaqueductal gray (PAG) ([Kingsbury et al., 2011](#)) and the amygdala ([Laberge et al., 2006](#)). Notably, the Social Behavior Network is a good example of remarkably conserved structures across vertebrate species that comprises six “nodes” (or brain regions of interest): the extended medial amygdala, the LS, the preoptic area, the anterior hypothalamus, the ventromedial hypothalamus and the midbrain ([Goodson, 2005](#); [O’Connell and Hofmann, 2012](#)). This conserved evolution is also seen in neural ligand and receptors distribution involved in social behaviors such as the nonapeptides of the vasotocin family (including the mammalian oxytocin and vasopressin and their homologs in other taxa), which also present variations shaped by different selection pressures ([Goodson, 2008](#); [O’Connell and Hofmann, 2012](#)).

Brain imaging studies have at times revealed robust inter-species variations in neural responses to comparable stimuli between species regarding the same emotions, in macaques and titi monkeys with the use of PET scans for example ([Rilling et al., 2004](#); [Maninger et al., 2017b](#)). We suggest that these differences may rely on the diversity of social systems with distinct species not having the same emotional response to similar stimuli, and thus potentially relying on differing neural substrates acquired through evolution ([Panksepp, 2011](#)).

In this review, we summarize imaging studies of complex social emotions in humans and animals; here, complex indicates a condition of not falling in the definition of basic emotions (Tracy and Randles, 2011) but rather existing as a construct of several basic emotions (Pedersen et al., 1994; Wang et al., 2020). Moreover, we try to link social emotions with neurohormones and neurotransmitters involved when possible. We then seek to explore how brain imaging might further allow us to study emotions, particularly in animals, and we explore whether animals and humans present similar patterns of brain activity in similar situations, eliciting social emotions as a result of their common evolutionary history. Finally, we conclude that there are striking similarities across species, as well as potential species-specific variation, and we demonstrate how comparative studies also shed light on the importance of the oxytocin network in the neurobiology of social emotions across mammalian species.

In the following article, abbreviations are listed in Table 1.

List of abbreviations.

Neural substrates of emotional process in social relationships

Bond creation and maintenance: Maternal love, romantic love, feeling of friendship

Romantic and maternal love

We start here with what is perhaps the most famous and most studied social emotion: love. Love has been examined in a variety of contexts, such as that of a mother and her infant (“maternal love”), or between two romantic partners (“romantic love”), which in both cases leads to the formation of an attachment (Ainsworth, 1978). In social species, attachment is often considered a key behavioral process in the life of an individual, especially mammals (Insel and Young, 2001). Romantic and maternal love are linked to reproductive success. Although mother-infant attachment is not necessarily lifelong, and while it is highly dependent on the parental care style of the species (see review: González-Mariscal and Poindron, 2002), the research on mother-infant attachment is interesting for the consideration of the onset of social bonds (Hazan and Shaver, 1987; Numan and Young, 2016). Moreover, it is subserved by similar mechanisms

as romantic love as pointed out by a very recent meta-analytic review of functional imaging studies ([Shih et al., 2022](#)), suggesting a common evolutionary origin. Indeed, bonding with a primary, parental attachment figure may be important for future attachment with a partner during adulthood ([Rogers and Bales, 2020](#); [Savidge and Bales, 2020](#)).

Mother-infant attachment

Pharmacological and lesion studies on mother-infant attachment were conducted in animals ([Insel and Young, 2001](#)), mostly in rats. These studies implicated the medial preoptic area (MPOA), the bed nucleus of the stria terminalis (BNST), and projection sites such as the lateral habenula and the ventral tegmental area (VTA) ([Insel and Young, 2001](#)). As an example, a pharmacological study relied on the experimental manipulation of oxytocin in the VTA and the MPOA and vasopressin in the MPOA, showing how these peptides in these brain regions facilitate the onset of maternal behavior in postpartum rats ([Pedersen et al., 1994](#)). For more examples in mammals, see the review of [Numan and Young \(2016\)](#). Also in birds (*Taeniopygia guttata*), there is evidence that the nonapeptide arginine vasotocin (the avian homolog of vasopressin) participates to attachment (search for proximity) between offspring and their parent ([Baran et al., 2016](#)). The avian homolog of oxytocin, mesotocin, is involved in the process of imprinting (i.e.,

attachment to a parental figure), by showing a higher preference for a stuffed hen in newly hatched chicks receiving intracranial mesotocin injections as compared to as vehicle (Loveland et al., 2019).

Therefore, in humans, researchers specifically examined regions rich in oxytocin (OT) and vasopressin (AVP) receptors (Loup et al., 1991; Freeman and Young, 2016) with fMRI when studying mother-infant attachment (Lorberbaum et al., 2002; Bartels and Zeki, 2004). Researchers utilized fMRI in humans to observe the brain activity of mothers while they looked at pictures of human faces, including their own infant, familiar children (a first control condition), and their best friend and other familiar adults (as additional controls). They first described striking similarities between maternal love and romantic love (from their previous study: Bartels and Zeki, 2000). Bartels and Zeki (2004) found that the induction of maternal and romantic love both activated regions that belong to the reward system (Bartels and Zeki, 2004), some of which may contain a significant density of oxytocin and vasopressin receptors in humans (e.g., substantia nigra for OTR and AVPR1a, and the globus pallidus and the ventral pallidum for OTR only; Loup et al., 1991; Frehner et al., 2022). Both types of love also activated the brain regions involved in the reward system (therefore implicating dopamine), as well as decreased activation of brain regions related to social judgment and regions associated with negative affect (Table 2). Maternal love also activated specific

areas that were not active during romantic love, such as the PAG, a region with oxytocin and vasopressin receptors, as well as the lateral orbitofrontal cortex (OFC) (Bartels and Zeki, 2004; Noriuchi et al., 2008). The involvement of the PAG in maternal love was subsequently confirmed by another fMRI study (Noriuchi et al., 2008).

Summary of the brain areas involved in the expression of “love” across species.

Comparing maternal love and related experiences of mother-infant attachment or affiliation in humans and rodents is challenging; however, one scenario where this emotion could presumably be expressed is when mothers are directly interacting with their pups. In rats, it is possible to induce the onset of maternal behaviors by repeated exposure to pups (Fleming and Rosenblatt, 1974). The neural activations are similar to that observed during episodes of human maternal love has been found using fMRI in nursing female rats. The suckling of pups activated the dopamine reward system, which may help reinforce the bond between the mother and the pup (Ferris et al., 2005). In spite of the variable sensory conditions between rodent and human studies (for instance, use of tactile stimuli in rats vs. psychological stimuli in human), neural activity converges on a common pathway, which is thought to involve reward. Infant cry studies in humans simulated separation of the mother from her infant, and were also conducted to study mother-

infant attachment. One of the first fMRI studies using infant cries to study mother attachment compared brain activations of mothers listening to their baby cries and white noise ([Lorberbaum et al., 1999, 2002](#)). The second study confirmed the implication of the BNST, the MPOA and the VTA in the maternal response to distress call, as well as the anterior cingulate cortex (ACC). The ACC has been implicated in both the neurobiology of the distress call from the infant, and in the response of the mother (see more in the section “Separation from a valuable relationship: Social pain, loneliness, and grief”).

Pair mate attachment and romantic love

Definitions of a pair bond across species generally require a selective association between two adults that contains an affective component and lasts outside of one reproductive cycle ([Bales et al., 2021](#)). Pair bonds as we understand them in mammals are latent psychological constructs that cannot be directly assessed, but which are operationalized by the presence of particular behaviors (e.g., maintenance of close physical proximity) in combination with an emotional component (e.g., attraction or arousal) ([Bales et al., 2021](#)).

As mentioned above, human volunteers that felt deeply in love were imaged with fMRI while observing a picture of their romantic partner (compared to a friend) ([Bartels and Zeki, 2000](#)). Men and women presented the same activation pattern in

the brain, and surprisingly, the authors described a limited number of areas that were activated: increased activity in the medial insula, in the ACC, and the caudate nucleus and the putamen, all bilaterally. In addition, deactivations (a decrease of the BOLD signal as compared to the control condition or negative BOLD response) were observed in the posterior cingulate gyrus, in the amygdala and were right-lateralized in the prefrontal, parietal and middle temporal cortices. These results are supported by their second study (Bartels and Zeki, 2004) focusing on brain regions known to contain high densities of oxytocin and vasopressin receptors (Loup et al., 1991). Deactivations were similar to the previous study (see also Table 2; Bartels and Zeki, 2004). They found that the VTA, the dentate gyrus/hippocampus and the hypothalamus were only activated by romantic love, which was not previously detected. Indeed, a following study on intense romantic love also found that the VTA (and the caudate nucleus) were very important in the formation of romantic bonds, by implicating the reward system (Fisher et al., 2005). This later cited study also points out a very important finding: they report that in couples with longer relationships (8–17 months as compared to new relationships), other brain regions had a higher BOLD signal, including the right mid-insular cortex, the right ACC and PCC, and the right posterior cingulate/retrosplenial cortex. As such, this suggests that the brain areas involved in romantic relationships evolve through time rather quickly. Four other fMRI

studies on passionate love were included in a meta-analysis and they were also consistent with the finding that love was recruiting areas linked with reward and emotions, but also social cognition, attention and self-recognition (Ortigue et al., 2010).

Presentation of a picture of a romantic partner, compared to a friend or highly familiar acquaintance, can be used to study the various aspects of love. For example, a group of Chinese participants was imaged using fMRI with the intention of detecting any divergences between Western and Eastern cultures (Xu et al., 2011). Indeed, it is problematic that most human studies cited above and below had been conducted on WEIRD participants (Western, Educated, Industrialized, Rich, and Democratic) or even STRANGELY WEIRD (people who interact with Social media, engage in Temporary relationships, can Relocate with relative ease, have Autonomy in mate choice, are Nulliparous, experience social Group segmentation, are being tested in an Educational setting, have Lots of options, and are Young adults) in this area (Henrich et al., 2010; Goetz et al., 2019). The Chinese participants also presented brain activation in dopamine-rich regions and mid-OFC, as well as a deactivation of the amygdala, the medial OFC and the medial accumbens. This work suggests that human culture does not affect the neural response in romantic love and is a reminder that diversity in the origins of participants should be considered more often in human studies. However, as

noted by the authors (Xu et al., 2011), the finding that medial OFC and the medial accumbens activity is decreased in the early stage of love conflicts with other findings in love, maternal love and friendship (Bartels and Zeki, 2004; Acevedo et al., 2012; Azzi et al., 2012; Watson and Platt, 2012; Brent et al., 2014a). The authors argue that the intensity and the direction of the fMRI activation/deactivation in a particular brain area is less relevant than the detection of a change as compared to a control condition.

A more recent study in humans also investigated which brain regions were associated with love maintenance (i.e., Eros change, defined as the difference of general couple satisfaction between the two time points) over the first year of bonding by identifying which parts were activated at the onset of a marriage and a year after (Acevedo et al., 2020). Love maintenance was associated with activation (increase of the BOLD signal) of a dopamine rich region, the SN and with the paracentral lobule (PCL) at the two time points, and with deactivation on the inferior frontal gyrus. This study confirmed the role of dopamine rich regions, and also the importance of oxytocin and vasopressin receptors in brain expression in a love maintenance context, as they also provide evidence of the interaction of vasopressin (AVPR1a rs3), oxytocin (OXTR rs53576), and dopamine (DRD4-7R) receptor gene variants with the activation of the VTA during the feeling of love. To our knowledge, there is currently no evidence of OXTR binding in the VTA in

primates, while it exists in rodents (Peris et al., 2017). Finally, the role of dopamine in romantic love in humans was also confirmed by a study using [¹¹C]raclopride, a dopamine D2/D3 receptor antagonist (Takahashi et al., 2015).

Most research on the neural substrates of pair bond formation, neuroimaging studies and otherwise, has been performed in rodents and humans. Here, we have primarily described neuroimaging studies in humans. While pair bonding is rare among non-human primates (NHP), a few species share this social organization with humans (Kleiman, 1977; Fuentes, 1998; Bales et al., 2021). Significant work examining the neural substrates of pair bond formation has also been performed in monogamous NHP, like titi monkeys, with the use of PET scans co-registered with MRI. In brief, conducting a PET scan on an NHP consist in injecting a radio-tracer (¹⁸F-glucose or any compound of interest) to the subject before a behavioral experiment during the radiotracer uptake period, where the subject is fully awake and unrestrained. After the uptake period, the subject is anesthetized and scanned to identify which brain regions had a higher radiotracer uptake during the behavioral experiment. For example, radiotracer uptake in male titi monkeys was compared before and 48-h after pairing with a female mate. The authors found differing glucose uptake in long-term pair bonded males than in lone males in specific brain areas related to bonding (Nacc, VP, MPOA, Amyg, SON, and LS),

indicating sustained differences in neural activity in those areas (Bales et al., 2007). More recently, in a study of pair bond formation in male titi monkeys, patterns of glucose uptake in short-term pair-bonding in PET scans were similar to brain areas commonly found in humans and rodents with other methods, specifically in the dopaminergic and motivational areas, as well as areas involved in social cognition (Maninger et al., 2017a).

Long-term attachment

How can love last a lifetime? Is love at its onset the same as after several years of bonding (Eastwick et al., 2019)? In a study of long-term attachment in humans (people married an average of 21.4 years), neural activation through fMRI showed many similarities with shorter-term studies, including increased activation in the reward system in response to images of romantic partners (Acevedo et al., 2012). Reported feelings of love correlated with the activation of the VTA and the caudate nucleus, while feelings of friendship correlated with a globus pallidus (GP) response. Interestingly, the left amygdala was active for long-term romantic love while it is deactivated for early stages of romantic love. In addition, a greater activation of the NAcc and the caudate were associated with the number of years of marriage and sex frequency with their partner (Acevedo et al., 2012).

Neural patterns associated with love in early stages of romantic relationships may predict the maintenance of feelings of love as relationships age. A follow-up experiment re-assessed the experience of love 40 months after an initial fMRI study on 18 participants; researchers investigated whether the neural patterns associated with a love-related stimulus at early stages of a relationship was predictive of later relationship happiness (Xu et al., 2012). The activity of certain brain areas (i.e., anterior medial OFC, right subcallosal cingulate, and right accumbens) at early stages of the relationship were negatively correlated with the scores of relationship happiness 40 months later, while there was a positive relationship between happiness 40 months later and the activity of the caudate tail and posterior medial OFC, implicating them in the maintenance of happiness in long-term relationships (Xu et al., 2012). However, this study did not conduct a second fMRI investigation at the second timepoints 40 months later in order to see whether these brain regions were still implicated at the later stage of the relationship.

In titi monkeys, twelve long-term paired males (i.e., around 1 year of pairing) and five unpaired males were compared using PET scans in order to determine which brain regions may be involved in the maintenance of long-term relationships (Bales et al., 2007): surprisingly, males in long-term pair-bonds had significantly lower relative uptake in the NAcc, VP, MPOA, medial amygdala, supraoptic nucleus of

the hypothalamus (SON), and LS than lone males. The lone males were subsequently paired and scanned after 48 h. As previously described in regard to short-term bonding, increases in glucose uptake were observed both globally and regionally in motivational and social memory areas following 1 week of pairing (Maninger et al., 2017a). These results observed in long-term bonding may seem to contradict those of the previously cited study (Bales et al., 2007). However, the issue is a methodological one. In the 2007 study, regional glucose uptake was normalized by dividing by whole brain uptake. However, the subsequent study found that whole brain uptake itself was up-regulated with pairing (Maninger et al., 2017a). When regional uptake was adjusted instead by injected dose of radiation, dopaminergic and social memory areas showed increases over and above those shown in whole brain glucose uptake.

Dopamine is also implicated in the long-term maintenance of pair bonds in titi monkeys. In one study, researchers used a dopamine receptor antagonist (D1) marked with a C¹¹ radiotracer to visualize and compare (*via* PET scan) D1 binding before pair bond formation and then 4–9 weeks (i.e., a long-term pairing) after pair bond formation in titi monkeys (Hostetler et al., 2017). Long-term pairing was associated with an increase in D1 binding, specifically in the LS. This particular finding is notable because in titi monkeys, the LS also has oxytocin receptors (Freeman et al., 2014b).

Imaging studies across species seem to converge to very similar findings about love and attachment, in humans, NHP, and rodents, implicating motivational areas involving dopamine and social areas involving oxytocin ([Table 2](#)), therefore supporting the existence of a “socially rewarding mechanism” underlying love (or similar affective states in non-humans) and attachment ([Preston, 2017](#)). Several moderating aspects have been identified, including effects of culture, relationship duration, and relationship type (e.g., maternal-offspring or mate-mate attachment). In contrast to the investigation of maternal and romantic love, there is a complementary line of research examining platonic love. Thus, while less researched, there is a body of literature that examines feelings and responses associated with friendship.

Emotions and affect associated with friendship

Friends are often used as control subjects in studies of love. The complex set of emotions expressed during a social situation involving a friend or a close social bond is something we refer to as a “feeling of friendship.” In animals, friendship is often referred to as a social bond, and the definition of a social bond is based on the quality of the relationship and the pattern of interactions between the two individuals: friends engage in bidirectional affiliative (non-aggressive, non-

reproductive) interactions at a higher rate and more consistently than non-friends (Brent et al., 2014a).

In human studies friends are often used as controls for studies focused on romantic love, although some studies have focused explicitly on friendship. In an fMRI study, brain activation elicited by friends was compared with that elicited by neutral family acquaintances (i.e., relatives that were variably familiar to participants, Acevedo et al., 2012). Activation of the posterior GP and the insula was identified during the feeling of friendship (Acevedo et al., 2012). Moreover, authors identified common activation in romantic love and platonic love in the medial OFC, the hypothalamus, the PAG and also in the left hemisphere of the cerebellum. In another human study (Güroğlu et al., 2008), the experience of friendship was not considered a social emotion but rather as an important experience that activates similar brain areas involved in empathy and reward expectancy. The authors compared the neural activity between three types of relationships (positive, negative, and neutral) and three types of items (peers, celebrities, and objects). They found higher activation in the amygdala and hippocampus, the NAcc, and the ventro-medial PFC when subjects interacted with their friends (i.e., positive peers) than with other peers and celebrities.

In a previous review on the neuroethology of friendship ([Brent et al., 2014a](#)), authors have suggested that the OFC in humans and NHPs plays an important role for social behaviors and bonding, and that social interaction may be intrinsically rewarding. For example, single neuron recording in the OFC indicated an activation associated with social cues ([Azzi et al., 2012](#); [Watson and Platt, 2012](#)) and motivational rewards in macaques. In another study in rhesus macaques, the ACC neuron activity was associated to reward allocation to another individual ([Chang et al., 2013](#)). However, to date, we have been unable to identify published references for whole brain imaging of non-human animal friendships.

Although friendship is seemingly less investigated than maternal or romantic love, the patterns of neural activation associated with friendship similarly imply activation of the reward system (see [Table 3](#)). In addition, animal models are particularly interesting for the study of friendship, as closeness can be similarly evaluated through social behavior in humans and animals. Friendship is also of interest because of its connection to the phenomenon of trust ([Fareri et al., 2020](#)). Notwithstanding that few studies have studied the neural substrates of the feeling of friendship, rewarding experiences and related brain activations are nevertheless increased in presence of a friend or a close social relationship ([Fareri et al., 2020](#)). Similar brain regions are activated when two friends are experiencing the same event ([Parkinson et al., 2018](#)), demonstrating some level of neural and affective

synchrony between friends and potentially the importance of social relationships in emotional processes. There is now additional compelling evidence of the importance of friendship for health (Dunbar, 2018). Relatedly, it is important to have a thorough understanding of the affective experiences of love and friendship under positive circumstances in order to then explore what may occur when important social bonds are disrupted or threatened.

Threat to a valuable relationship: Jealousy

Jealousy is an emotional response to a perceived threat to a valuable relationship, and it is a complex emotion that is generally characterized as constructed of several basic emotions, such as fear of loss, anxiety, suspiciousness, and anger about betrayal (Cubicciotti and Mason, 1978; Parrott and Smith, 1993). The response to the threat can elicit various behaviors, such as proximity seeking or aggressive behaviors depending on sex and species (as we will see below). For example, jealousy may be associated with violence toward the partner in men but also in women (de Weerth and Kalma, 1993; Harris, 2003). The jealousy response in humans can be associated with psychological factors such as self-esteem and how much the relationship is threatened (Sharpsteen, 1995) as well as emotional dependency (Buunk, 1982).

In humans and animals, it is possible to create social situations that should elicit jealousy and to observe behaviors or self-reported affective experience that indicate the elicitation of jealousy. While it is still easy in humans to confirm the induction of jealousy with self-report measures (Takahashi et al., 2006; Steis et al., 2021), studying jealousy in animals requires a specific stimulus situation intended to jeopardize the valuable relationship (Winslow et al., 1993; Rilling et al., 2004; Maninger et al., 2017b; Cook et al., 2018; Webb et al., 2020). A jealousy scenario is, for example, a situation in which a potential new bond could be formed between a third individual (or stranger) and one of two members of an established pair-bond. One advantage of using animal models here is the possibility to simulate more realistically a situation where the bond is in danger while still maintaining some control over the consequences. Typically, in human studies, jealousy-inducing scenarios (or vignettes) are proposed by the experimenter to participants so that the participants can imagine themselves in said jealousy-inducing situation; or alternatively, participants are engaged in a *Cyberball* game where they experience social exclusion during a game (Zheng et al., 2021). During this game, participants virtually exchange a ball between two other (fake) players— the participants are led to believe that the other players are real people, when in fact participants are playing with a computer. At some point, the other players stop playing with the participant, thus simulating social exclusion. In the studies

presented below, the protocols were specifically designed to elicit and study jealous reactions in humans and animals, and they provide a clear mention of this goal.

In humans, two types of jealousy have been defined in the contexts of social relationships with a romantic partner: sexual jealousy, involving sexual infidelity; and emotional jealousy, involving a loss of investment from the partner in the relationship (Buss et al., 1992; Buss and Haselton, 2005). It is also commonly alleged that men are more prone to sexual jealousy and women to emotional jealousy (Buss et al., 1992), which can be explained from an evolutionary perspective: sexual infidelity carried out by the female partner could lead the male partner to invest in paternal care for an infant/child that is not his own; and emotional infidelity on the part of the man could lead to a decreased investment in existing offspring of the couple. This explanation is notably heteronormative, and it may overemphasize maternal reliance on or concern about paternal care in a cooperatively breeding context in which alloparental care is potentially available. Nevertheless, these two “jealousy-types” have been distinguished in the human brain using fMRI (Takahashi et al., 2006), such that both types of jealousy were investigated in men and women responding to a questionnaire and undergoing an fMRI while thinking about scenarios of infidelity. While self-report measures indicated that men and women felt equally jealous for both types of infidelity (i.e.,

both sexual and emotional), brain imaging revealed distinct patterns of brain activation between sexes (Takahashi et al., 2006). In women, the posterior STS showed an increased activation correlated with the rating on emotional jealousy, and other cortical regions and the thalamus. The posterior STS is commonly involved in interpretation, deception, trustworthiness, and violation of social norms (Winston et al., 2002). However in men, insula, cortex and thalamus activation were detected and correlated to the rating on emotional jealousy. The amygdala and the hypothalamus (implicated in appraisal of sexual salience and reproductive behavior) were also implicated in male sexual jealousy as shown by the increase in BOLD signal in the study of Takahashi et al. (2006). Both men and women showed an activation of the visual cortex while thinking about sexual infidelity and of the visual cortex and the thalamus while thinking about emotional infidelity. This study is an important reminder of potential sex effects in the neural bases of emotion, even when self-report does not appear to differ by sex and/or gender. In addition, a more recent fMRI study focusing on women that suffered from sexual infidelity by their romantic partner presented a larger spectrum of brain activations when listening to the description of their experience than in the previous cited study (insula, ACC, PCC, mPFC, substantia nigra, GP, nucleus subthalamicus, and hypothalamus). It is interesting to note that in this more “ecologically” relevant scenario (with an actual experience of sexual infidelity), the pattern of activation is

not only larger, but also includes regions involved in men jealousy (thalamus) and male monkeys (insula, PCC, and ACC), showing the importance of the experimental design to elicit the desired emotional responses in human subjects.

To date, we have identified only two other imaging studies on the neural basis of jealousy in NHP (Rilling et al., 2004; Maninger et al., 2017b) and one in dogs (Cook et al., 2018). In male rhesus monkeys, researchers elicited a behavioral reaction in individuals seeing their consort, a receptive female from his social group, with another male in the jealousy condition or alone in the control condition. Notably, while not characterized as socially monogamous in contrast to other NHP like titi monkeys, macaques still form temporary social relationships during the estrus of the female. In male titi monkeys, subjects viewed a stranger male in proximity to a stranger female in the control condition, and a stranger male in proximity to the subject's pair mate (Maninger et al., 2017b). Behaviorally, macaques presented more aggressive behaviors during the jealousy condition without a significant increase or decrease in stress or affiliative behaviors. In contrast, titi monkeys displayed more lip-smacking behavior (an affiliative behavior) in the jealousy condition and showed higher plasma cortisol and testosterone. In a PET scan following the presentation of a jealousy/control situation, macaques that responded more intensely to the jealously inducing stimulus presented right activation of the STS and the right amygdala, the right

cerebellum, and a bilateral activation of the insula. While the right hemisphere is often associated with negative stimulus in humans, the STS, and the insula are associated with increased vigilance, face-processing and judgments of trustworthiness. In titi monkeys, PET scan imaging revealed a higher activation of the right LS, the left PCC and the left ACC, and interestingly, a decrease in the right amygdala, during the jealousy condition. This contrasts with the study in male macaques (Rilling et al., 2004) and in jealousy in men (Takahashi et al., 2006), both of which found an increase in right amygdalar activation.

Patterns of brain activation associated with jealousy in male macaques are comparable to patterns identified in human women (Takahashi et al., 2006): namely the activation of the right STS, which was related to increased vigilance in humans (see Table 4). They were also comparable to human men in the activation of the right amygdala. The insula, which has been associated in humans with the judgment of trustworthiness and perception of visceral responses to emotional stimuli, was activated in jealous male macaques but not in jealous humans. During the control condition, male macaques showed a greater relative activation in the thalamus, left precentral gyrus, left cuneus, left amygdala, and right cingulate sulcus. The authors interpret the left activation of the amygdala as a positive attraction to the female. Interestingly, this particular activation of the left amygdala during the control condition was correlated with the activation of the right

amygdala during jealousy. The activation of the amygdala can be associated with jealousy in humans, macaques and dogs, and can be related to aggressive behavior (Cook et al., 2018). In dogs kept still in an MRI scanner, subjects in a jealousy-inducing condition reacted with higher activation of the amygdala when witnessing their caregiver giving food to a fake dog, in contrast to dogs in a control condition in which their caregiver simply placed food in a bowl (Cook et al., 2018). Notably, the authors of this study warn that the amygdala activation should not be automatically associated to only one specific emotion, but rather to a higher state of arousal.

Summary of the brain areas involved in the expression of jealousy across species.

It is necessary to note that there is a significant difference in the methodology used in the respective, aforementioned studies in macaques and titi monkeys: i.e., the titi monkeys were shown a stranger male in both conditions whereas the macaques observed a stranger male only in the jealousy condition (Rilling et al., 2004; Maninger et al., 2017b). One interpretation could be that the presence of a stranger male could activate the right amygdala in macaques in the jealousy condition, but in contrast, since the stranger male is present in both conditions with the titi monkeys, the decrease in the right amygdala could be due to the positive presence of the pair-bond. This hypothesis is empirically supported, as titi

monkeys did not display more aggressive behaviors (in contrast to the aggression demonstrated by macaques), but rather higher levels of lip smacking, an affiliative behavior probably directed to reinforcement of the pair bond. To test this, one might consider the inclusion of an additional control in future studies, i.e., the presentation of the female partner alone as for titi monkeys. However, as explained by the authors of both articles, for time, technical and financial reasons, all controls could not be tested with the same individuals. While higher plasma cortisol and testosterone in jealousy condition advocate in the favor of a jealous reaction, titi monkeys do not seem to react in an aggressive manner during jealousy. Rather, the activation of a specific brain area, the LS, an area rich in oxytocin, vasopressin and dopaminergic receptors in titi monkeys (Freeman et al., 2014b; Hostetler et al., 2017), suggests that mechanisms reinforcing bonding are activated during this challenging condition for this species.

One evolutionary interpretation of these findings in animals and humans would be that jealousy motivates an individual to try to “save” the existing relationship with their pair mate from disruption (Panksepp, 2010; Maninger et al., 2017b), but the behaviors that are involved are species-dependent. The sex differences shown in humans also clearly highlight the importance of studying both sexes when possible. Taken together, these studies demonstrate some similarities and some variation across species (Table 4), that might be partially related to the changes in

experimental designs that need to be adapted to the species. These studies on jealousy are also a reminder that each emotion involves neural systems rather than one specific region.

Separation from a valuable relationship: Social pain, loneliness, and grief

A word on emotional loneliness and social loneliness

In humans, social separation or loss are associated with emotions like loneliness and social pain (Eisenberger, 2006), but both terms are at times used interchangeably. Emotional loneliness in humans is typically assessed by questionnaires (for example the UCLA-LS, Russell et al., 1978) and sometimes with the use of the *Cyberball* game according to a recent review on the neurobiology of loneliness (Lam et al., 2021). Because these methods can only be used in humans, here, we focus primarily on studies of animals in which brain imaging was combined with scenarios like short-term and long-term social separation from valuable social partners, and we rely more on the term “separation distress.” Social separation is known to elicit depressive behavior in mice (Martin and Brown, 2010), and multiple NHP species (Worlein, 2014), so here, we also

acknowledge that animals can potentially experience affective states akin to loneliness as well.

Social pain

Separation distress, or “social pain” is defined as a distressing experience due to the perception of a psychological distance and can be interpreted from an adaptive perspective as a process to promote social contact to strengthen relationships. For example, separation distress in maternal-infant bonds is viewed as a mechanism that elicits distress behaviors (e.g., calls) from the infant and maternal behavior from the mother, thus protecting the infant from estrangement and potential dangers of being alone (Eisenberger, 2006). Lesion studies in non-human mammals have first highlighted the role of the ACC, the anterior insula and the PAG in distress vocalizations (Eisenberger, 2012). Squirrel monkeys placed in isolation vocalized less when their ACC was ablated (MacLean and Newman, 1988). The electrical stimulation of the ACC produces vocalization in rhesus macaques (Smith, 1945). Rat pups separated from their mother show less distress call vocalization when the PAG is lesioned, and stimulation of the PAG can lead to spontaneous distress calls (Panksepp, 1998).

While the invasive studies cited above have pointed out the importance of the ACC in distress vocalization in pups and animal infants, the ACC has also been

implicated in the neural response of the mother receiving the calls. Indeed, distress calls received by a mother separated from her infant elicit maternal reactions in animals and humans (Fleming and Rosenblatt, 1974; Ainsworth, 1978). As mentioned before (in the mother-infant attachment section) comparing the fMRI of mothers listening to their own infant cry or white noise revealed not only the activation of the ACC, but also other regions associated with maternal love and attachment (MPOA, VBNST, and VTA) (Lorberbaum et al., 1999, 2002).

Brain regions detected by fMRI for reactions related to social pain in a *Cyberball game* include the ACC and the right ventral prefrontal cortex (RVPFC) in humans (Eisenberger et al., 2003). An extensive body of work on human brain activation (reviewed in Eisenberger, 2012) advocates for a central role of the dorsal ACC and the anterior insula during social and physical pain, while the dorsal insula and the anterior insula are also activated when participants reported feeling of being excluded. The activation of the dorsal ACC is also modulated by parameters like friendship or self-esteem, which highlights how much physical pain and social pain rely on a similar neural basis (Eisenberger and Lieberman, 2004; Eisenberger, 2012). Interestingly, the dorsal ACC and the anterior insula which are activated during social pain, present a reduced activation when participants take pain killers such as acetaminophen (DeWall et al., 2010). However, there has been some criticism regarding the use of the *Cyberball* paradigm to study social rejection. One

criticism is the hypothesis that any negative emotion or affective state may activate the ACC (*reviewed in Eisenberger, 2015*). Also, a recent meta-analysis on *Cyberball* studies found no evidence for the activation of the dorsal ACC during social exclusion (*Mwilambwe-Tshilobo and Spreng, 2021*), but that rejection engaged the default network instead.

One study on the topic of romantic rejection has employed an alternative paradigm in lieu of the *Cyberball* game, in which participants viewed a picture of themselves next to a second (fake) participant along with accompanying feedback from the second participant, noting if the second participant liked the real participant or not. Researchers identified an activation of the ventrolateral prefrontal cortex (vlPFC) and the anterior insula (*Hsu et al., 2020*), especially in men. However, this study also mentioned one limitation similar to the *Cyberball* game, which is the use of a fake participant as a stimulus. Studies with real participants as stimuli are rare (as reviewed in *van der Watt et al., 2021*), but these paradigms also generally engage the cingulate and the prefrontal cortex.

Until recently, little was known about the neural basis of separation anxiety in humans. An fMRI study in healthy human adults positively correlated *a priori* reports of higher levels of separation anxiety with a higher level of amygdalar activation in response to viewing negatively valanced faces (*Redlich et*

al., 2015). However, this study did not directly image the neural response of anxiety during separation, but rather a correlation between an earlier, self-reported separation anxiety score during questionnaires and the later brain response during threatening face viewing. Authors also acknowledged that stimuli consisted of faces of strangers rather than those of close acquaintances; nevertheless, this study remains one of the rare examples of studies designed to determine the neural basis of separation anxiety in humans using fMRI. In another study, researchers compared healthy mothers to mothers with interpersonal violence-related posttraumatic stress disorder (PTSD) to assess neural activity while viewing their respective children in separation as compared to another child (Schechter et al., 2012). Mothers with PTSD showed decreased activation of the superior frontal gyrus and middle frontal gyrus. The comparison between the two studies is difficult given the two contexts (stranger threatening/neutral faces or own/stranger child) and given the differences in the methods (correlation of fMRI with anxiety score or direct analysis of fMRI), but they both converge in the sense that the amygdala and the superior frontal gyrus are implicated in negatively valenced stimulus (Schechter et al., 2012; Redlich et al., 2015).

Loneliness

Loneliness can be conceptualized as a form of social pain, and an ultimate, evolutionary origin for loneliness has been proposed (Cacioppo and Cacioppo, 2018). This recent evolutionary theory posits that loneliness is an adaptive mechanism that improves survivability of individuals found in a socially isolated situation by increasing motivation to look for social connection with other individuals and by increasing vigilance. According to this theory, neural mechanisms associated with motivational processes (including those that engage OT and dopamine) should be involved in loneliness as well as aversive responses (e.g., vigilance for social threats). A recent review highlighted the neural basis of loneliness in human subjects in studies using various structural/functional imaging and other non-imaging methods including computer tomography, MRI/fMRI, electroencephalography, diffusion tensor imaging, single-photon emission computed tomography, PET scans, and post- mortem brain tissue RNA expression or pathological analysis (Lam et al., 2021). Many of the studies on loneliness used the University of Los Angeles Loneliness Scale, a questionnaire designed to measure how much participants felt lonely. The collection of reviewed literature ultimately connected the feeling of loneliness with several brain areas, e.g., the PFC, the anterior insula, the amygdala, the hippocampus, and the posterior superior cortex. Interestingly, the PFC and the insula were also implicated in social pain,

but there are relatively few mentions of the ACC found in studies on human social rejection in this particular review on loneliness.

Short-term separation in animals

The study of separation differs from that of loneliness in that it is a response to the loss of a specific individual. Short-term separations in young rhesus monkeys from their mother followed by a PET scan showed that the right dorsolateral PFC and the right ventral temporal/occipital lobe were both activated by maternal separation, and that these regional activations were positively correlated with cortisol level (possibly indicating a state of stress) (Rilling et al., 2001).

Conversely, activity of the left dorsolateral PFC decreased with separation (Rilling et al., 2001). In titi monkeys (Hinde et al., 2016), researchers instead focused their investigation on the cingulate cortex because of *a priori* evidence from human studies of grief (Gündel et al., 2003) and social pain (Eisenberger, 2015); and authors also investigated regions in the reward system (i.e., the NAcc and VP), which were previously associated with grief in humans and prairie voles (O'Connor et al., 2008; Bosch et al., 2016). Researchers separated male titi monkeys from their female partner for 48 h and collected measures of neural activity from the males with PET imaging (Table 5). Following separation, imaging indicated decreases in glucose uptake in a number of regions (LS, VP,

PAG, PVN of the hypothalamus, and cerebellum, Table 5) and an increase in CSF OT, plasma cortisol, and insulin (Hinde et al., 2016). The authors conclude that an increased release of OT and binding to OTR in the LS, and OT to AVPR1a in the PAG and cerebellum, could represent a potential mechanism of dealing with social separation and a preparation to the encounter of a new mate or an adaptive response to create a new bond quickly.

Long-term separation or loss

Bereavement or grief in humans may occur following a permanent loss and is and is expressed in various cultures (Eisenbruch, 1984). Studies of grief in humans present bereaved persons with visual stimuli depicting a deceased individual or alternatively with a verbal evocation. In women who had lost a first degree relative in the past year, PCC, medial/superior frontal gyrus, and cerebellum were activated by picture or verbal evocation of the deceased relative (Gündel et al., 2003). A variety of other regions were specifically increased by one stimulation or the other (see Table 5), and pet owners who have experienced loss of their animal also show activations of regions linked with sadness (Freed et al., 2009). Women who experienced the death of a mother or sister in the past 5 years and who have accordingly experienced complicated grief also show increased activation of the reward system (namely in the NAcc, O'Connor et al., 2008). These authors posit

that this activation of the reward system may interfere with the process of adapting to the loss in the present.

Following a long-term (i.e., 2-week) period of social separation, PET imaging in male titi monkeys reflected a decrease in glucose uptake in the central amygdala and in the whole brain, as well as an increased CSF OT and increased plasma insulin concentrations (Hinde et al., 2016). This increase in CSF OT and insulin are interpreted as a sign of motivation for engaging in social interaction. The reduced, global glucose uptake of the brain associated with long-term separation seems to reflect a reverse process that contrasts with the increased, global glucose uptake which normally occurs during pair bond formation (Bales et al., 2007; Maninger et al., 2017a). Thus, there is mixed evidence: on one hand there appears to be activation of physiological processes meant to encourage social interaction; on the other hand, there are also processes at play that may reflect a process of adaptation to partner loss. In addition, titi monkey fathers that encountered a new stranger female showed lower glucose intake in the SON and the PVN, as well as a lower PCC uptake, when compared to non-fathers. When reunited with their pair-mates, fathers showed higher plasma cortisol concentrations, and lower CSF OT, plasma AVP and glucose concentrations than non-fathers. This higher PCC glucose uptake in non-fathers is interpreted as a potential higher openness/interest in a novel female, since the activation of the

PCC has been implicated in the process of human partner choice (Yokoyama et al., 2017). This additional result reflects the need to consider the effects of other social factors and relationships that can modulate the response in brain activations between subjects as mentioned before in humans, but also that the emotions elicited by the loss of a bond might be mixed with the will or adaptation to create a new bond.

Some regions are regularly found to be activated by social pain (unrelated to death) in humans, which can be elicited, for example, by separation, loss or exclusion from a group (Eisenberger, 2006), namely the dorsal and ventral ACC and the anterior insula (as extensively reviewed by Rotge et al., 2015) and the rostro-ventral PFC (see Table 5). It is still complex to disentangle social pain, grief and separation in animals; however, the comparison between human and non-human species reveals striking similarities, therefore suggesting common evolutionary roots.

Discussion, limitations, and further research

Are there commonalities *between species* in the neural basis of social emotions?

For that matter, are there neural commonalities *between emotions* that are related to being social? While it has been proposed (and we have discussed it here) that certain emotions are specifically social, there are a number of different perspectives on this issue. At least one empirical study failed to find a specifically social, fundamental dimension for emotion ([Bliss-Moreau et al., 2020](#)). Brain imaging studies of social emotion in animals, while still in their early days, have already provided critical insights into similarities and variation between species, studying a variety of social bonds and affective states with adapted imaging and emotion assessment methods ([Figure 1](#)), and have highlighted the implication of several brain areas ([Table 6](#)). Several considerations arise when considering the role that imaging can play in future studies on social emotion and the understanding of its evolution.

Unifying mechanisms for social emotions

One unifying mechanism for social emotions may be the actions of the neurohormone OT across mammalian species and across emotions, particularly in the instances of love or social separation, although vasopressin is also likely to play a role ([Panksepp, 2010](#); [Carter, 2014, 2017](#)). The social salience hypothesis of OT ([Shamay-Tsoory and Abu-Akel, 2016](#)) suggests that OT's focuses attention on social cues and social contexts, and OT mediates responses through interactions with the dopaminergic system. Neural systems involved in the production of or response to OT and dopamine thus make a reasonable place to look for common neural bases of emotion, and many of the studies cited here have done so.

One complication is that for many NHP species, the location of OT receptors is still not known ([Freeman and Young, 2016](#)). In fact, distributions have been published only for common marmosets ([Schorscher-Petcu et al., 2009](#)), rhesus monkeys ([Freeman et al., 2014a](#)), titi monkeys ([Freeman et al., 2014b](#)), and recently, several species of lemurs ([Grebe et al., 2021](#)) and chimpanzees ([Rogers Flattery et al., 2021](#)). There is relatively little overlap in these receptors between NHP species, although the overlap is much higher when the closely related vasopressin system is also considered ([Freeman and Young, 2016](#)). Attention should be paid to our basic knowledge of the OT system in each species, when

considering what neural changes tell us about the neurobiology of emotion in that species.

Evolutionary perspectives relative to social system

Non-human primates not only share a close evolutionary history with humans, but also present a large variety of social systems that could theoretically allow the study of how social system has impacted the evolution of emotions. However, the paucity of brain imaging studies in NHP allows us to draw few conclusions at this point. Monogamous titi monkeys showed similar engagement of brain structures as humans did during love, for instance, but showed some key differences during jealousy. Currently, it is often not possible to tell whether these distinct results are due to specificities of the social system, individual variation, or methodological differences. In future, many more species need to be studied, and experimental conditions should be matched as closely as possible to allow greater comparability between studies.

To aid in comparing studies, we recommend sharing protocols data on brain imaging of social emotions ([Milham et al., 2020](#)). Indeed, it would allow analyses with larger sample sizes and homogenize practice in the brain imaging analysis field that lack reproducibility ([Poldrack et al., 2012](#)). In primatology, researchers have proven their ability to work in a collaborative way, as for example in

cognition with the ManyPrimates project ([Altschul et al., 2019](#); [ManyPrimates et al., 2019, 2020](#)).

Primatologists focused on cognition have demonstrated a large effect of phylogeny over the effect of any ecological or social factor, at least for short-term memory, in an extremely large number of species ([ManyPrimates et al., 2019](#); [ManyPrimates, 2021](#)). Given the tight relationship between cognition and emotion ([Gosling, 2001](#); [Kremer et al., 2020](#)), one could reasonably hypothesize that the way affect and emotions are encoded in the brain is also highly constrained by phylogeny. This would mean that more closely related species have more comparable patterns of brain activations under the same condition than more distantly related species. This viewpoint is supported by the results on jealousy, in which jealous humans and macaques (more closely related) are more similar to one another than either are to more distantly related titi monkeys. An alternative (but not necessarily contradictory) perspective is that the social system of a species (e.g., monogamy or cooperative breeding) may promote similarity between phylogenetically distant species by means of convergent evolution. It is certainly possible for both explanations to be true in different situations.

Finally, we highlighted many non-human primate and mammal studies in this review, mainly because we were targeting brain-imaging studies, which were

mainly conducted in these taxa. It is also important to keep in mind the importance of a larger range of taxa to be able to conduct a comparative analysis of social emotional responses to better understand its evolution. Indeed, vertebrate brains present a remarkable conservation of their Social Decision-Making Network (O'Connell and Hofmann, 2012), which could be important for social emotions like empathy (Tremblay et al., 2017). In addition, concerning the OT/AVP system and its relationship with the social system, a very important comparative work has been conducted in a subset of five finch species, all monogamous but who live in different group size: the comparative study of these example of species suggested that the evolution of the nonapeptide system could have been the main driver of social system convergence, leading to changes in group sizes in some species (Goodson and Kingsbury, 2011). Some evidence also suggest that, convergently with mammals, the role of the nonapeptides have the same function for offspring care in birds (Goodson et al., 2012).

Neurochemical specificity

A significant, missing piece of information associated with measuring glucose uptake in PET scans is the identification of specific neurotransmitters involved in the activation of neurons within a specific brain region. However, it is also possible to use PET scans to explore these mechanisms more closely. As it has been

proposed, PET scan studies can be conducted with specific radiotracers in order to target neurotransmitters that are circulating in the brain ([Hostetler et al., 2017](#)). For example, the use of a D1-receptor antagonist can detect the upregulation of D1-receptors in the brain after long-term pairing in titi monkeys ([Hostetler et al., 2017](#)). While radiotracers for some receptor systems (such as oxytocin and corticotropin-releasing hormone) remain elusive ([Smith et al., 2016](#)), tracers for other systems (dopamine, opioids, serotonin) have been used in humans and would mostly just require validation for the animal species being studied. In addition, pharmacological manipulation can be used in conjunction with imaging and behavior to give additional insight ([Shamay-Tsoory and Abu-Akel, 2016](#)).

Lack of studies on females

A general caveat encountered in animal biology is the study bias toward males. This is especially the case in neuroscience in which many studies exclusively examine male subjects or participants. While this problem is less severe in human studies, 5.5 studies in males were reported for each study in females in animal neuroscience in year 2009 ([Beery and Zucker, 2011](#)). This disparity seems to have ameliorated over the past decade ([Woitowich et al., 2020](#)). Behavioral responses may at times be sexually dimorphic. For example, in monogamous prairie voles, females and males do not show the same patterns of neural or behavioral processes

during pair bond formation and separation (Lieberwirth and Wang, 2016). In the case of jealousy, both studies that we reviewed on primates were conducted on males alone. One particular reason for that may be due to the fact that female monkeys may either demonstrate less jealousy-associated behavior, as is the case for titi monkeys (Cubicciotti and Mason, 1978); or alternatively, for female monkeys in a species that presents a polygynous social system, jealousy may be ecologically irrelevant (e.g., female macaques) (Thierry et al., 2004). However, given the complexities in attributing emotion in animals, the possibilities of feeling emotion without overt display of behavior, and the possibility of sex-specific neural substrates for similar emotions, studying these questions in both sexes should become standard.

Choosing control conditions across studies

One challenge that becomes apparent in a review of the emotion and affect literature is the difficulty in choosing an appropriate or comparable control condition. Brain imaging is a relatively non-invasive procedure that allows one to test individuals in several tests and control conditions; however, brain imaging studies are very expensive, and it can prove financially burdensome or prohibitive to cultivate a large sample size (to capture variability) while simultaneously establishing several test conditions. Moreover, there are ethical limitations that

preclude the imposition of too many stressful manipulations/experiments on any one individual. For this reason, researchers, in lieu of controlling for various conditions (e.g., alone or with the mate, with a stranger, or with a mate), instead generally select one relevant control condition. Clearly, more time and financial resources, as well as more coordinated studies, are needed to support brain imaging to standardize results in comparative studies on social emotion imaging in non-human animals. In general, brain imaging studies in animals acknowledge the difficulties encountered in choosing perfect control conditions as for the example in jealousy (Rilling et al., 2004; Maninger et al., 2017b). To cultivate conditions of jealousy or an appropriate control, the subject must perceive a threat to a valuable social relationship (Webb and de Waal, 2018) or the absence of that threat, respectively. These circumstances may be achieved by removing the presence of a competitor or, alternatively, by removing the valuable social stimulus. Ultimately, it is critical to establish a control condition that does not induce unwanted, extraneous social factors, for example additional stress or anxiety due to social isolation. Social isolation may be particularly important for some primate species, depending on their social system.

All emotions are not investigated equally

While there is a relatively large body of work on romantic love and maternal love, especially in humans, there are many fewer brain imaging studies on other social emotions, for instance the feeling of friendship or jealousy, in either humans or animals. Jealousy studies in contexts other than consort or mate relationships, such as friendships or parent-infant relationships, are also very rare ([Webb et al., 2020](#)); even though jealousy is a social emotion that might be expressed very early by young children ([Panksepp, 2010](#)). Being able to target desired emotions in animals is difficult and relies on the identification of emotions that appear to be relevant for a given species, and accordingly, this research depends on creating appropriate stimulus situations that should evoke the desired emotion. For instance, how researchers should best study grief in animals remains challenging. We cannot assess the certainty of knowledge that an animal has about the loss of a social companion. We believe that these losses in animals that have attachment relationships could lead to a state like human grief. However, given our state of uncertainty we will refer to “loss” rather than “grief.” Other potential behavioral indicators of social emotion, such as “slow blink” in cats ([Humphrey et al., 2020](#)) or tail-wagging in dogs, could help us to access emotions like “trust” or perhaps even love in additional species.

Other social emotions associated with specific behaviors are regularly reported in primates and in pet dogs (Morris et al., 2008; de Waal, 2019), such as shame, pride (Tracy and Randles, 2011) or even the sense of fairness and inequity, and they represent an enormous and exciting field of investigation to explore using brain imaging technologies. Perhaps another complicated emotion related process to study would be empathy. Empathy has been described as our ability to be affected by the emotional state of another (de Waal, 2008), although other definitions have been argued. To our knowledge, neuroimaging studies on empathy or affect contagion have been published in humans but not in animals (Platek et al., 2005; Decety and Grèzes, 2006). If one is to acknowledge the presence of emotion or affect in animals, there is perhaps no reason to deny the presence of empathy or related processes in animals; and, subtle experimental designs are needed to image empathy and related processes (e.g., affect or stress contagion). For example, which regions of the brain are activated when a macaque or a chimpanzee observes a conspecific yawning (Anderson et al., 2004; Paukner and Anderson, 2006)? Do patterns of neural activation of dogs reflect the process of affect contagion or empathy for other dogs?

In humans, empathy of social pain has been imaged in humans looking at other people being socially excluded, with highly empathic people also exhibiting patterns of brain activation associated with social pain (anterior insula and dorsal

ACC) ([Masten et al., 2011](#)). Yet, only a few experiments have been attempted involving animals (and not always mentioning empathy *per se*). Rodent species can show helping behaviors and consolation behavior, so they also seem to be good models for the study of the physiology and neurological basis of empathy and have helped to describe the implication of oxytocin and of the ACC in this emotion related process ([Burkett et al., 2016](#); [Cox and Reichel, 2021](#); [Mason, 2021](#)). A human-animal fMRI study has been conducted with humans attributing emotions to humans and to animals ([Spunt et al., 2017](#)); the same neural mechanisms were involved in the attribution of emotions in humans and animals, involving dorsomedial and lateral orbitofrontal prefrontal cortices. Such experiments could be further investigated in animals.

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